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# THE DETERMINATION OF AMMONIA AND AMIDE NITROGEN IN TOBACCO BY THE USE OF PERMUTIT.*

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The accurate determination of the preexisting ammonia or of the amide nitrogen of tobacco is rendered difficult by the volatility of nicotine which distils over more or less completely when the standard procedures for the estimation of these forms of nitrogen are employed. A number of attempts has been made to devise methods whereby corrections for the nicotine content of such distillates may be calculated but none of these is entirely satisfactory.

Kissling (1) distilled the volatile bases into standard acid, and subtracted from the titration figure the equivalent of the nicotine determined upon another sample by ether extraction. This method for the estimation of the ammonia was shown by Kissling (2) to include some amide nitrogen. It may also include other volatile bases. Harrison and Self (3) removed the nicotine from a similar distillate by precipitation with potassium triiodide reagent and again distilled the ammonia. This method takes no account of the possible presence of other volatile bases. Kissling (4) in 1920 removed the nicotine from the sample by ether extraction and subsequently distilled the ammonia. This method was shown to be inaccurate by Fodor and Reifenberg (5) because some of the ammonia passes into the ether extract. These authors removed the nicotine from the distillate by precipitation with a solution of mercuric iodide in potassium iodide and subsequently distilled the ammonia. This method is open to the same objections as that of Harrison and Self and all of these procedures are time-consuming.

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In the investigation of the tobacco leaf now being carried on in this laboratory it has become necessary to develop accurate methods for the determination of the simpler forms of nitrogen which can be applied to the green leaf as well as to manufactured tobacco. Existing methods for the estimation of nicotine leave little to be desired on the score of accuracy or convenience. A method for the determination of nitrate nitrogen has been developed and forms the subject of a recent paper (6). In the present communication a simple and accurate method to determine amide nitrogen and preexisting ammonia nitrogen is described. It is based on the observations that nicotine is absorbed by permutit only to a very small extent and gives no appreciable color with Nessler's reagent, whereas Folin and Bell (7) have shown that ammonia can be quantitatively removed from a faintly acid solution by permutit, can subsequently be set free from the permutit by alkali, and determined by Nessler's method. The data in a paper by Whitehorn (8) indicate that many basic substances, including nicotine and several of the volatile amines, undergo extensive base exchange with permutit. It has been found, however, that neither nicotine, which is abundant in tobacco extracts, nor trimethylamine, which may possibly be present, interferes with the determination of ammonia under the conditions that we have employed.

*Behavior of Solutions of Methylamines and of Nicotine towards Permutit.*

Our observations have led us to believe that tobacco extracts contain a small proportion of a volatile base in addition to ammonia and the alkaloids that are precipitated by silicotungstic acid. In view of the possibility that this substance may be one of the methylamines the behavior of monomethylamine, dimethylamine, and trimethylamine toward permutit was studied under conditions suitable for the quantitative removal of ammonia from solution by this reagent. In order to remove traces of ammonium chloride the monomethylamine hydrochloride was crystallized twice from absolute alcohol, the other two amine hydrochlorides from chloroform. The nicotine was crystallized twice as picrate from water and the free base was subsequently distilled *in vacuo*.

The aqueous solutions of the basic hydrochlorides (10 to 20 cc.)

were shaken with 2 to 2.5 gm. of permutit for 3 minutes and the permutit was then washed four times by decantation with 15 to 25 cc. portions of water. The washings were collected and distilled with alkali into standard acid for the determination of the amine content; nicotine was determined by the silicotungstic acid precipitation method. The data are given in Table I, and indicate that the proportions of the three amines taken up by permutit decrease with increase in their molecular weight. Only a small proportion of the nicotine reacted with permutit.

TABLE I.

*Base Exchange Relations of Methylamines and of Nicotine with Permutit.*

| Substance. | Free base taken. | Free base recovered. | Taken up by permutit. |
|---|---|---|---|
| | *mg.* | *mg.* | *per cent* |
| $CH_3 \cdot NH_2HCl$ | 14.6 | 2.6 | 82 |
| | 14.6 | 2.5 | 83 |
| $(CH_3)_2NH \cdot HCl$ | 20.0 | 10.2 | 49 |
| | 20.0 | 9.4 | 53 |
| $(CH_3)_3N \cdot HCl$ | 20.0 | 14.2 | 29 |
| | 20.0 | 14.6 | 27 |
| | 19.9 | 14.6 | 27 |
| Nicotine $\cdot 2HCl$ | 16.84 | 15.6 | 7.4 |
| | 16.84 | 15.6 | 7.4 |

Monomethylamine, when present in quantities in excess of 5 mg. per 100 cc., gives a yellow turbidity with Nessler's reagent. At lower concentrations no turbidity is formed and the amount of color is too small to interfere with the colorimetric estimation of ammonia in the concentrations we have employed. Dimethylamine gives a little color with Nessler's reagent and, if present in more than small amounts, might give rise to high results for ammonia. Neither trimethylamine nor nicotine gives colors with Nessler's reagent which interfere with the estimation of ammonia. In Table II are given data to illustrate the accuracy with which ammonia may be determined in the presence of the.methylamines or of nicotine. In these experiments the ammonia was taken up on permutit, the solution containing the amine was decanted

off, the permutit was washed four times with water and the ammonia was then set free from the permutit by sodium hydroxide. A colorimetric determination of the ammonia was then made by the technique more fully described below. The results indicate that *ammonia can be determined with a considerable degree of accuracy in the presence of these volatile bases by the use of permutit and of Nessler's reagent.*

TABLE II.

*Determination of Ammonia in Presence of Methylamines and of Nicotine*

| Base | Free base taken | Ammonia N taken | Ammonia N found |
|------|------|------|------|
| | mg | mg | mg |
| $CH_3NH_2 \cdot HCl$ | 2 | 0 30 | 0 31 |
| | 5 | 0 30 | 0 32 |
| | 5 | 0 50 | 0.51 |
| | 10 | 0 30 | Turbidity. |
| $\begin{array}{c}CH_3 \\ CH_3\end{array}\!\!>\!\!NH \cdot HCl$ | 5 | 0 30 | 0 30 |
| | 5 | 0 30 | 0 31 |
| | 5 | 0 20 | 0 21 |
| | 5 | 0 20 | 0 21 |
| | 10 | 0 30 | 0 33 |
| | 10 | 0 30 | 0 33 |
| $\begin{array}{c}CH_3 \\ CH_3 \\ CH_3\end{array}\!\!\to\!\!N \cdot HCl$ | 20 | 0 30 | 0 30 |
| | 20 | 0 30 | 0 31 |
| | 20 | 0 50 | 0 51 |
| | 20 | 0 50 | 0 50 |
| Nicotine $\cdot$ 2HCl | 17 | 0 30 | 0 29 |
| | 17 | 0 30 | 0 28 |
| | 20 | 0 50 | 0 48 |
| | 20 | 0 50 | 0 48 |

### Determination of Ammonia in Tobacco Extracts.

The ammonia contained in tobacco extracts can be quantitatively removed from them by direct treatment with permutit provided these are so diluted that the concentration of the inorganic salts is too low to interfere with the reaction. This procedure is, however, inadvisable since the correct dilution cannot readily be ascertained and the pigments of the extract are also

taken up to some extent. It is therefore preferable first to distil the ammonia into acid and then subject the distillate to the permutit treatment. The ammonia is subsequently liberated by alkali and determined colorimetrically by Nessler's reagent.

## Reagents.

(a) Ammonium sulfate stock solution; 2.358 gm. per liter, preserved with chloroform. 2 cc. = 1.0 mg. of nitrogen.

(b) Ammonium sulfate standard solution prepared by diluting 200 cc. of the stock solution to 1000 cc. and preserving with chloroform. 1 cc. = 0.1 mg. of nitrogen.

(c) Nessler's solution prepared according to Folin (9).

(d) Sodium hydroxide, 10 per cent aqueous solution.

A quantity of an extract of tobacco containing between 0.25 and 1.5 mg. of ammonia nitrogen is placed in a 300 cc. Kjeldahl flask together with water to make 25 to 30 cc. A few angular quartz pebbles, a small piece of paraffin, and from 1 to 2 gm. of heavy magnesium oxide are added and the flask is fitted for distillation as described by Folin and Wright (10). The distillation tube is dipped beneath the surface of 3 cc. of 0.1 N hydrochloric acid containing a drop of methyl red solution in a 50 cc. centrifuge tube; the contents of the flask are mixed and heated to boiling with a micro burner at such a rate that steam begins to rise from the receiver in about 3 minutes. Distillation is continued for 5 minutes, the tube is then removed, the end is washed with a few drops of water, and the distillate is cooled. A sufficient number of 100 cc. volumetric flasks are charged with 2.5 to 3.0 gm. of permutit prepared according to Folin and Bell's directions and the permutit is washed several times by decantation with distilled water. To three of the flasks are transferred 10 cc. of water, 3 cc. of 0.1 N hydrochloric acid, 1 drop of methyl red solution and 0.3, 0.5, and 1.0 mg. respectively of ammonia nitrogen as standard ammonium sulfate solution. The distillates are transferred to flasks containing washed permutit together with enough water to make approximately 15 cc.; all the flasks are shaken for 5 minutes and laid on their sides for 1 minute; the fluid is then decanted and the permutit is washed by decantation three times successively with 10 to 20 cc. of water, settling for 1 minute each time. The permutit is rinsed to the bottom of each flask with about 5 cc.

of water, 1 cc. of 10 per cent sodium hydroxide is added, and the
flasks are shaken 3 minutes; about 65 cc. more water are added,
the flasks are agitated, and 10 cc. of Nessler's reagent are added to

TABLE III

*Recovery of Added Ammonia Nitrogen from Tobacco Extracts*

| Extract | Ammonia N taken | Ammonia N found | Recovered |
|---------|-----------------|-----------------|-----------|
| *cc* | *mg* | *mg* | *per cent* |
| 20 | 0 20 | 0 194 | 97 |
| 20 | 0 20 | 0 192 | 96 |
| 20 | 0 20 | 0 198 | 99 |
| 20 | 0 20 | 0 188 | 94 |
| 20 | 0 20 | 0 190 | 95 |
| 5 | 0 24 | 0 22 | 92 |
| 3 | 0 30 | 0 285 | 95 |
| 3 | 0 30 | 0 283 | 94 |
| 5 | 0 50 | 0 47 | 94 |
| 5 | 0 50 | 0 48 | 96 |
| 2 | 0 50 | 0 47 | 94 |
| 10 | 1 00 | 0 95 | 95 |
| Average . | | | 95 |

TABLE IV

*Influence of Free Acid upon Base Exchange of Ammonia with Permutit.*
Volume of solution extracted = 20 cc   Ammonia nitrogen = 0 50 mg.

| Acid taken | Ammonia N found | Recovered |
|------------|-----------------|-----------|
| | *mg* | *per cent* |
| 5 cc  0 1 N | 0 500 | 100 |
| 5 "  0 1 " | 0 498 | 99 6 |
| 5 "  0 1 " | 0 490 | 98 0 |
| 5 "  0 1 " | 0 495 | 99 0 |
| 5 "  0 2 " | 0 481 | 96 2 |
| 5 "  0 2 " | 0 481 | 96 2 |
| 5 "  0 4 " | 0 395 | 79 0 |
| 5 "  0 4 " | 0 400 | 80 0 |
| 5 "  1 0 " | 0 220 | 44 0 |

each.  The contents are diluted, mixed, and compared in a colori-
meter with the nearest standard.  The intensity of the colors
does not change for several hours.

The short distillation with magnesia under the above conditions is sufficient to remove ammonia quantitatively from extracts. Data are given in Table III which show that ammonia added to tobacco extracts can be recovered with an average accuracy of 95 per cent. The 0.1 N hydrochloric acid in which the distillate is collected does not interfere with the subsequent base exchange with permutit. In Table IV are given data showing that the ammonia recovery is markedly depressed only when more than 5 cc. of 0.2 N hydrochloric acid are present; there is therefore a considerable margin of safety when 3 cc. of 0.1 N acid are used. The treatment of the standard solutions with permutit is not essential but it contributes to the over-all accuracy of the method.

TABLE V.

*Determinations of Ammonia Nitrogen in Different Extracts of Tobacco.*

| Extract No. | Ammonia N found. | Extract No. | Ammonia N found. |
|:---:|:---:|:---:|:---:|
| | *mg.* | | *mg.* |
| 1 | 0.269 | 3 | 0.268 |
| | 0.271 | | 0.266 |
| | 0.266 | | 0.266 |
| | 0.273 | | |
| | 0.275 | 4 | 0.298 |
| | | | 0.293 |
| 2 | 0.270 | • | |
| | 0.271 | | |

A series of experiments in which known quantities of 0.3 to 1.0 mg. of ammonia were carried through the permutit process and compared with similar quantities of standard ammonium sulfate solution showed that 97.1 to 98.1 per cent of the ammonia could be recovered. This small but constant error is avoided when both standard and unknown are treated alike.

Amides do not interfere with the ammonia determination. When 5.0 mg. of pure asparagine were distilled with magnesia as described the color obtained in the distillate with Nessler's reagent was very slight and corresponded to the presence of 0.01 to 0.03 mg. of ammonia nitrogen. Thus, unless extraordinarily large proportions of asparagine are present in a plant extract, no inaccuracy is to be apprehended. Arginine, which is slowly decomposed by hot alkali with the production of ammonia, is not

notably attacked under the above conditions.  A quantity of
300 mg. gave less than 0.01 mg. of ammonia nitrogen when distilled
with magnesia for 5 minutes.

In addition to making possible the rapid and accurate determina-
tion of ammonia in the presence of nicotine and of trimethylamine,
the present procedure possesses a distinct advantage over the
customary direct distillation of ammonia with magnesia into a
standard solution of acid.  The titration of such distillates,
whether secured from plant extracts or from protein hydrolysates,
is usually difficult owing to the presence of substances produced
by the action of hot alkali which interfere with the accurate
observation of the end-point.  Although this difficulty can be
avoided by conducting the distillation *in vacuo*, with barium or
calcium hydroxide and alcohol, an elaborate apparatus is necessary
and the procedure is time-consuming.  Moreover larger amounts
of the material are required than can sometimes be spared.

In Table V are given data upon four different tobacco extracts
illustrating the reproducibility of the determinations of ammonia.

## Determination of Amide Nitrogen.

Amide nitrogen in plant extracts is usually determined by the
method of Sachsse (11) which depends upon the fact that aspara-
gine, the amide most commonly occurring in plants, is completely
hydrolyzed to aspartic acid and ammonia by boiling for 3 hours
with 4 per cent hydrochloric acid.  Amide nitrogen may be
conveniently determined in tobacco extracts as follows.  A
quantity of extract of known preformed ammonia content which
will contain between 0.2 and 1.0 mg. of ammonia nitrogen after
hydrolysis is boiled under a reflux condenser for 4 hours with 1 cc.
of 5 N hydrochloric acid for each 5 cc. of extract employed.  The
hydrolysate is then transferred to a 300 cc. Kjeldahl flask and
ammonia is determined as previously described.  The increase
in ammonia found after hydrolysis gives a measure of the amide
nitrogen of the extract.  The data in Table VI illustrate the
accuracy of the method as applied to pure asparagine and those in
Table VII show the proportion of asparagine added to tobacco
extracts that was recovered.  The accuracy of the amide nitrogen
determination is somewhat less than that of the ammonia nitrogen;
this is to be expected since the proportion of amide nitrogen is

calculated from the difference in ammonia content before and after hydrolysis and therefore contains the error of the ammonia determination twice over.

TABLE VI.

*Recovery of Amide Nitrogen from Pure Asparagine Solutions.*

| Amide N taken. | Amide N found. | Recovered. |
|:---:|:---:|:---:|
| *mg.* | *mg.* | *per cent* |
| 0.21 | 0.22` | 105 |
| 0.30 | 0.29 | 97 |
| 0.50 | 0.47 | 94 |
| 0.50 | 0.50 | 100 |
| 0.50 | 0.50 | 100 |
| 0.50 | 0.496 | 99 |
| 1.00 | 0 95 | 95 |
| 1.00 | 0.97 | 97 |
| Average................... .................... | | 98.4 |

TABLE VII.

*Recovery of Amide Nitrogen of Asparagine Added to Tobacco Extracts.*

| Amide N taken. | Amide N found. | Recovered. |
|:---:|:---:|:---:|
| *mg.* | *mg.* | *per cent* |
| 0.05 | 0.047 | 94 |
| 0.20 | 0.18 | 90 |
| 0.30 | 0.27 | 90 |
| 0.30 | 0.29 | 97 |
| 0.30 | 0.29 | 97 |
| 0 30 | 0 29 | 97 |
| 0.30 | 0.28 | 93 |
| 0.30 | 0.26 | 87 |
| 0.30 | 0.27 | 90 |
| 0.50 | 0.46 | 92 |
| 1.00 | 0.90 | 90 |
| Average.............:.... ..................... | | 92.5 |

Although the above methods were developed primarily for the determination of ammonia and amide nitrogen in tobacco; that is, in the presence of nicotine, they should prove useful for the investigation of other plants. Our knowledge of the volatile

bases of most plant tissues is limited; in general the total volatile base is titrated and reported as ammonia. The occurrence of trimethylamine in the cotton plant (12), and possibly also in alfalfa (13), suggests that this practice may not always be justifiable.

## SUMMARY.

A method for the determination of preformed ammonia and of amide nitrogen in tobacco is described. The ammonia is distilled from the untreated or hydrolyzed sample according to the technique of Folin and Wright, is taken up on permutit, and is subsequently determined colorimetrically by the use of Nessler's reagent; it has been shown that nicotine does not interfere. The base exchange relationships of monomethyl-, dimethyl-, and trimethylamine with permutit have been studied. None of these amines interferes with the determination of ammonia. The method is simple and rapid and can readily be employed in the investigation of other tissues.

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# PURINE METABOLISM.

## II. THE EFFECT OF THE INGESTION OF GLYCINE ON THE EXCRETION OF ENDOGENOUS URIC ACID.

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It has been generally accepted that the ingestion of high protein diets leads to an increased excretion of endogenous uric acid. In his review on purine metabolism, Rose (1) has summarized the important contributions on this phase of the subject and has also discussed the various theories which have been formulated to explain the observed phenomena. Lewis, Dunn, and Doisy (2) have made a careful study of the hourly elimination of uric acid by fasting individuals following the ingestion of proteins and amino acids. Definite increases in uric acid excretion were observed in all cases with a maximum effect 3 to 4 hours after the ingestion of protein and 2 to 3 hours after the ingestion of amino acids. The increased uric acid elimination was considered by these authors to be due to an increased production of uric acid, as a result of the stimulating action of the absorbed amino acids. The authors have cited the specific dynamic action of proteins and protein derivatives as a parallel phenomenon. In the experiments just cited the hourly elimination of creatinine was practically constant. Zwarenstein (3) was of the opinion that if there was a general increase in cellular metabolism, there should also be an increase in creatinine elimination coincident with the increase in uric acid. Zwarenstein, therefore, repeated the work of Lewis, Dunn, and Doisy. His results indicated that the hourly excretion of uric acid by fasting individuals was not affected by the ingestion of proteins and amino acids.

Inasmuch as these results are at variance with all preceding work since 1905, it was thought advisable to make a study of the ex-

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perimental procedures employed by Zwarenstein. The only significant point of difference was in the method used for the determination of uric acid. Lewis, Dunn, and Doisy (2) as well as other workers (4, 5) on this problem have precipitated the uric acid from the urine as an insoluble salt prior to its determination by a colorimetric procedure. Zwarenstein (3) on the other hand has used the more recent direct colorimetric method of Benedict and Franke (6). In this method the urine is treated with suitable reagents for the development of the uric acid color without the preliminary precipitation.

TABLE I.

*Effect of Presence of Amino Acids on Determination of Uric Acid by the Benedict-Franke Direct Method.*

| Uric acid present. | Glycine present.* | Amino acid N present. | Uric acid found. |
|---|---|---|---|
| *mg.* | *mg.* | *mg.* | *mg.* |
| 0.2 | 0 | 0 | 0.2 |
| 0.2 | 0 25 | 0.046 | 0.174 |
| 0.2 | 0.50 | 0.093 | 0.152 |
| 0.2 | 1 00 | 0.187 | 0.136 |
| 0.2 | 2.00 | 0.373 | 0.121 |

\* The amounts of glycine indicated in the table were added directly to the flasks containing the standard amount of uric acid, prior to the development of the color.

It is very probable that there would be an increase in the amino acid content of the urine following the ingestion of proteins and amino acids in the amounts employed by Lewis, Dunn, and Doisy (2) and Zwarenstein (3). This might be expected particularly for the urines collected during the first 2 or 3 hours after the ingestion of the proteins and amino acids. These, it will be recalled, were the periods in which Lewis, Dunn, and Doisy observed an increased uric acid excretion. Therefore as a preliminary step, it was thought advisable to study the effect of added amino acids upon the direct determination of uric acid by the Benedict-Franke method. Accordingly, equal amounts of a standard uric acid solution (0.2 mg. of uric acid) were measured into a series of 50 cc. volumetric flasks. One flask was retained as a standard, while to each of the other flasks a definite amount of a glycine solution

was added. The contents of the flasks were all made up to the same volume (11 cc.) prior to the addition of the reagents, which develop the color. The determination was completed exactly as described by Benedict and Franke (6). It was obvious, even before the final solutions were compared in the colorimeter, that the presence of the added amino acid had prevented the full development of color of the uric acid. This experiment was repeated several times and while the results were not identical they are of the same order. The results of a typical experiment are presented in Table I. It is apparent from this table that the presence of even small amounts of amino acid nitrogen (0.046 mg.) leads to considerable error in the uric acid determination by the method of Benedict and Franke (6).

To determine whether an increased concentration of amino acids in the urine would influence the determination of uric acid by the Benedict-Franke method, urines were analyzed before and after the addition of glycine. The same urines were then reanalyzed for uric acid by the method of Morris and Macleod (7). In the latter method the uric acid is separated from the urine as the zinc salt, prior to its colorimetric estimation. The results of several experiments clearly indicated that if the analysis for uric acid was made by the direct method of Benedict and Franke, the presence of 1 mg. of added glycine led to results which were from 20 to 25 per cent too low. The effect of the added amino acids was negligible, however, if the analysis was made by the method of Morris and Macleod. The results of two experiments are given to illustrate this point. The analysis of a urine indicated a concentration of 0.43 mg. of uric acid per cc. of urine by the method of Benedict and Franke and 0.46 mg. per cc. by the method of Morris and Macleod. Glycine was then added to a portion of the urine so that the amount of urine used for a second analysis contained 1 mg. of added glycine (0.187 mg. of amino nitrogen). For the second analysis, a value of 0.34 mg. of uric acid per cc. was obtained by the method of Benedict and Franke and 0.45 mg. by the method of Morris and Macleod. A second urine on analysis gave a value of 0.40 mg. of uric acid per cc. by both methods. In the presence of 1 mg. of added glycine, the value for uric acid by the Benedict-Franke method dropped to 0.29 mg. per cc.,

and the value obtained by the method of Morris and Macleod was 0.38 mg. per cc.

These preliminary experiments suggest that Zwarenstein's failure to find an increase in uric acid elimination following the ingestion of proteins and amino acids might be explained by analytical difficulties. In the experiments reported by Zwarenstein, Subject A ingested 10 gm. of glycine in the first experiment and 20 gm. of alanine in the second experiment. Subject B consumed 250 gm. of boiled egg white in one experiment and 200 gm. of Cheddar cheese in a later experiment. As suggested previously in this paper, it is probable that the ingestion of such large amounts of proteins and amino acids would lead to an increased excretion of amino acids in the urine. A small increase in the amino acid content as shown would cause considerable error in the uric acid determination by the Benedict-Franke method. No data are available in the literature, however, relative to the hourly excretion of amino nitrogen under the conditions of Zwarenstein's experiments. The authors of this paper, therefore, decided to repeat part of the work of Zwarenstein, analyzing the hourly samples of urine for amino acid nitrogen as well as uric acid and creatinine.

In two experiments the urines were analyzed for uric acid by the method of Benedict and Franke (6) and by the method of Morris and Macleod (7). In a third experiment the uric acid was determined by the above methods, and also by the method of Folin and Denis (8) and the method of Benedict and Hitchcock (9). Thus, in this experiment, the uric acid was determined by one direct method and by three indirect methods, each of which employs a different method for the precipitation of the uric acid prior to its colorimetric estimation. Creatinine was determined by Folin's microcolorimetric method and amino acid nitrogen by the colorimetric procedure of Folin (10).

The experiments were carried out in the manner first described by Lewis, Dunn, and Doisy (2). The subject of the experiments was a healthy male, 65 kilos in weight, who lived on his usual diet on the days preceding the experiment. The usual evening meal was eaten at 6 p.m. on the day preceding the experiment and then no further food was eaten until the experiment was completed. On arising on the experimental day, the bladder was emptied, and

the collection of hourly samples began.  200 cc. of water were ingested at the beginning, of each hour to facilitate the collection of hourly samples of urine and to insure reasonably large volumes. After the collection of two control samples, 10 gm. of glycine were ingested and the hourly collection continued for the following 5

TABLE II.

*Effect of Ingestion of Glycine on Uric Acid Excretion.*

| Time. | Volume. | Creati-nine. | Uric acid. | | Amino acid N. | Amino acid N in sample analyzed.* |
|---|---|---|---|---|---|---|
| | | | Morris-Macleod method. | Benedict-Franke method. | | |
| Experiment 1; March 7. | | | | | | |
| | *cc.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 7–8  a.m. | 39 | 72 | 23.6 | 17.2 | 5.7 | 0.07 |
| 8–9    " | 40 | 71 | 21.2 | 17.4 | 5.2 | 0.07 |
| 9–10  "† | 60 | 76 | 27.6 | 18.7 | 27.9 | 0.23 |
| 10–11  " | 139 | 71 | 28.8 | 15.0 | 60.5 | 0.87 |
| 11 a.m.–12 m. | 133 | 68 | 27.0 | 15.6 | 24.2 | 0.36 |
| 12 m.–1 p.m. | 125 | 61 | 18.4 | 13.8 | 10.6 | 0.17 |
| 1–2 p.m. | 116 | 73 | 18.1 | 14.3 | 8.4 | 0.14 |
| Experiment 2; March 14. | | | | | | |
| 7–8  a.m. | 34 | 80 | 19.5 | 15.6 | 6.8 | 0.10 |
| 8–9    " | 29 | 63 | 15.7 | 12.9 | 5.0 | 0.09 |
| 9–10  "† | 50 | 67 | 27.0 | 11.5 | 27.5 | 0.55 |
| 10–11  " | 62 | 64 | 28.0 | 12.5 | 34.7 | 0.56 |
| 11 a.m.–12 m. | 50 | 68 | 21.0 | 10.3 | 15.5 | 0.31 |
| 12 m.–1 p.m. | 64 | 68 | 14.7 | 11.9 | 10.6 | 0.17 |
| 1–2 p.m. | 122 | 61 | 12.2 | 11.1 | 12.2 | 0.20 |

* By amino acid N in sample analyzed is meant the amount of amino acid nitrogen contained in the volume of urine used for the determination of uric acid by the Benedict-Franke method.

† 10 gm. of glycine ingested at 9 a.m.

hours.  It is to be noted that this study is limited to the effect of the ingestion of glycine upon uric acid elimination.  Our justification for this will be discussed later in the paper.  The results obtained are presented in Tables II and III.  The values for uric acid recorded in these tables have been carefully checked and rechecked by two observers.  The amounts of urine used

for the determinations of uric acid were such that the colorimetric readings fell between 15 and 25 with the standard set at 20 mm. Zwarenstein calls attention to the fact that this precaution was observed in his work. Unfortunately, this precaution does not prevent considerable error in the uric acid determination by the Benedict-Franke method, if the urines contain appreciable amounts of amino acid nitrogen. This will be discussed in detail later in the paper.

TABLE III.

*Effect of Glycine Ingestion on Uric Acid Excretion.*
Experiment 3; March 19.

| Time. | Volume. | Creatinine. | Uric acid. | | | | Amino acid N. | Amino acid N in sample analyzed.* |
| | | | Morris-Macleod method. | Benedict-Franke method. | Folin-Denis method. | Benedict-Hitchcock method. | | |
| | cc. | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
| 8–9 a.m. | 29 | 71 | 23.9 | 20.1 | 22.6 | 17.4 | 4.8 | 0.04 |
| 9–10 " | 28 | 69 | 23.1 | 19.0 | 18.5 | 15.4 | 4.4 | 0.041 |
| 10–11 "† | 50 | 71 | 32.8 | 19.0 | 30.8 | 32.0 | 27.5 | 0.27 |
| 11 a.m.–12 m. | 48 | 66 | 32.6 | 18.7 | 34.6 | 33.6 | 27.8 | 0.29 |
| 12 m.–1 p.m. | 47 | 67 | 25.4 | 16.9 | 26.3 | 25.4 | 12.9 | 0.14 |
| 1–2 p.m. | 43 | 73 | 20.6 | 15.7 | 16.8 | 21.5 | 6.5 | 0.08 |
| 2–3 " | 45 | 82 | 16.2 | 14.9 | 12.6 | 19.8 | 4.5 | 0.05 |

\* See foot-note to Table II.
† 10 gm. of glycine ingested at 10 a.m.

An examination of Tables II and III reveals the following points. (1) If the analysis for uric acid is made by an indirect method (Morris and Macleod, Folin and Denis, or Benedict and Hitchcock), there is a marked increase in the hourly excretion of uric acid for the three periods following the ingestion of glycine. (2) If the analysis for uric acid is made on the same urines by the direct method of Benedict, no increase over the control periods is observed. The values would indeed indicate a decrease for these periods. (3) For the first two or three periods following the ingestion of 10 gm. of glycine, the amount of amino acid nitrogen

excreted per hour is 5 to 6 times greater than that of the control periods.

In the last column of Tables II and III is given the amount of amino acid nitrogen present in the volume of urine which is used for the determination of uric acid by the Benedict-Franke method. A comparison of these values with the amounts of amino acid nitrogen which were shown to produce considerable error in the determination of uric acid by this method (see Table I) will explain why the uric acid values by the Benedict-Franke method are low for the experimental periods.   Our results then are in accordance with the work of Lewis, Dunn, and Doisy; that is, the ingestion of glycine produces a distinct rise in the uric acid excretion. It was not considered necessary for the purpose of this study to repeat the work with other amino acids or proteins since these results with glycine clearly indicate that Zwarenstein's results are at variance with those of earlier investigators because of the method he employed for uric acid.   Experiments have been carried out with alanine and glutamic acid similar to the experiment with glycine reported in Table I.   When equivalent amounts of these amino acids (glycine, alanine, glutamic acid) are present, the effect on the uric acid estimation by the Benedict-Franke method is much the same.

A few statements should be made regarding the uric acid values obtained by the Benedict-Franke method in Experiments 1 and 2 (Table II).   The amount of urine used for each determination was such that the colorimetric readings were in most cases between 16 and 20 mm. with the standard set at 20 mm.   In the light of results obtained in Experiment 3 (Table III), it is now believed that, if still smaller amounts of these urines had been used, the reading would still have remained in the colorimetric range (less than 25 mm.) and correspondingly higher results for uric acid would have been obtained.   The higher values for uric acid result from a dilution of the amino acids of the urine to a concentration which has less effect on the determination of uric acid by the Benedict-Franke method.   This point is well illustrated by the data taken from the analysis of the urine for the 11 to 12 o'clock period (Experiment 3, Table III).   In the first analysis of this urine by the direct Benedict-Franke method, 10 cc. of a 1:10 dilution were used.   The reading of the unknown against the

standard (0.2 mg. of uric acid) was 16.7 mm.   This on calculation yields a concentration of 0.24 mg. of uric acid per cc. of urine or 11.5 mg. for the hour period.   The urine was then reanalyzed by the same method, 10 cc. of a 1:20 dilution being used.   The colorimetric reading was 20.5 against the standard set at 20 mm.   This corresponds to a concentration of 0.39 mg. of uric acid per cc. of urine, or a total of 18.72 mg. of uric acid for the period as compared with 11.52 when twice as much urine was used.   A third analysis was made on 10 cc. of a 1:40 dilution of the urine, and the colorimetric reading was now 30.8 mm. against the 20 mm. standard.   This, of course, is outside of the range recommended for accurate work.   The amount of uric acid per period is 24.96 mg. if the calculation is made from the last determination.   It is to be noted that higher values for uric acid were obtained as the concentration of the amino acids decreased.   Even when 10 cc. of a 1:40 dilution of the urine were used, the amino acid nitrogen present (0.145 mg.) was sufficient to cause an error of 20 per cent as indicated by the preliminary experiments.   The uric acid excretion for this period as determined by the method of Morris and Macleod was 32.6 mg., by the method of Folin and Denis 34.6 mg., and by that of Benedict and Hitchcock, 33.6 mg.

### SUMMARY.

In confirmation of the work of Lewis, Dunn, and Doisy (2) these results show that the ingestion of glycine by a fasting man is followed by an increased hourly excretion of uric acid.   This effect is noted during the 1st hour after the ingestion of the glycine and continues for the following 2 hours.   It is believed that the failure of Zwarenstein (3) to obtain similar results under comparable experimental conditions was due to the use of the direct method of Benedict and Franke (6) for the determination of uric acid.   Experiments are given in this report, which show that the concentration of amino acids in the urine for the periods immediately following the ingestion of glycine (10 gm.) is from 5 to 6 times that for the control periods.   It has also been shown that the uric acid method of Benedict and Franke gives results which are much too low for the urines containing these higher concentrations of amino acids.

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# IS COPPER A CONSTITUENT OF THE HEMOGLOBIN MOLECULE?

## THE DISTRIBUTION OF COPPER IN BLOOD.*

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In a previous publication (1) from this laboratory dealing with the importance of copper as a supplement to iron for hemoglobin building in the rat, the question of the function of copper in this relation was discussed very briefly. The following statement was made: "So far as the literature shows, hemoglobin does not contain copper."

The presence of copper in blood has been reported by a few workers. McHargue (2) reported a variation of 1 to 4 mg. of copper per kilo of fresh blood taken from several different animals. Warburg and Krebs (3) found 1.7 mg. of copper per kilo of human blood serum. No reports are found in the literature concerning careful investigations of the presence of copper in pure hemoglobin.

## Copper in Hemoglobin.

Because no studies have been made directly on this pigment and because the possible presence of copper in the molecule is the first question to be answered in the determination of the function of copper in hemoglobin building, we undertook the following analysis of hemoglobin.

The first samples analyzed were furnished by Dr. W. C. Stadie, of the University of Pennsylvania, to whom we wish to express our thanks for this cooperation. Sample 1 was a preparation of oxyhemoglobin made from horse blood and purified by electro-

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dialysis.   Sample 2 was a similar sample except that the crystals were washed until the conductivity of the supernatant liquid was 2.7 times $10^{-5}$ ohms.   The method used for the determination of copper was that outlined by Elvehjem and Lindow (4) for the analysis of materials high in iron.   The analysis of Sample 1 gave 0.034 mg. of copper and Sample 2 gave 0.019 mg. of copper per gm. of dry hemoglobin.

These samples were also analyzed spectroscopically by Dr. Jacob Papish, Professor of Spectroscopy at Cornell University, to whom we wish to express our gratitude for these determinations.   Dr. Papish reported the presence of copper in both samples of hemoglobin, but did not estimate the quantity.

If we assume that there is 1 atom of copper per molecule of hemoglobin then the smallest molecular weight calculated from the percentage of copper found would be 1,870,588 for Sample 1 and 3,344,368 for Sample 2.   The recent work of Svedberg and Fahraeus (5) has placed the molecular weight of hemoglobin at 66,800.   The calculated figures are 25 to 50 times this value. Consequently the hemoglobin molecule cannot contain copper unless the present figures for the molecular weight are very much too low.   Another fact which indicates that copper is not a part of the hemoglobin molecule is that the sample purified to the largest extent contained the smallest amount of copper.

Because these samples of hemoglobin were prepared from horse blood and the original work demonstrating the importance of copper was conducted with rats, we felt that similar analyses should be made upon hemoglobin prepared from rat blood.

The blood was obtained from large adult rats by bleeding from the carotid artery.   The oxyhemoglobin was prepared according to the method of Heidelberger (6).   Since salt-free oxyhemoglobin was not desired, the preparation was not dialyzed.   Two samples of oxyhemoglobin were prepared.   The copper content of one sample was found to be 0.013 mg. of copper per gm. of oxyhemoglobin and the other 0.017 mg. of copper per gm. of hemoglobin, or an average of 0.015 mg.

On calculating the molecular weight from this figure, a value of 4,240,000 is obtained, which is again much greater than the accepted early figure of 16,700, or the later value of 66,800 reported by Svedberg and Fahraeus.   The method of Heidelberger for

the preparation of oxyhemoglobin is so mild in its procedure that it does not seem necessary to theorize that in this method of preparation the copper might possibly have been removed from the hemoglobin molecule. Although the small amount of copper found in the hemoglobin may be accounted for by assuming that the precipitate of oxyhemoglobin carried the copper down with it and was a contaminant, this is not necessarily the case. During the preparaton and analysis, several reagents were used. These reagents were shown to be copper-free by individual tests, but the possible accumulation of minute amounts of copper from the several sources may have been sufficient to account for the very slight test obtained.

### Distribution of Copper in Blood.

Reports regarding the distribution of copper in blood are also very meager. Besides the work of Warburg and Krebs, figures have been published by Abderhalden and Möller (7) and McHargue, Healy, and Hill (8). Abderhalden analyzed only the blood serum and found 1.9 mg. of Cu per liter of serum obtained from horse blood. He oxidized the serum with sulfuric and nitric acids, precipitated the copper from the solution with $H_2S$, and weighed the copper as copper sulfide. McHargue and coworkers found 0.0044 per cent of copper in dry serum and 0.007 per cent of copper in the dry cells and fibrin prepared from cow blood. These figures are equivalent to approximately 3.3 mg. per liter of fresh serum and 2.5 mg. of Cu per liter of undried corpuscles.

Because we had analyzed the hemoglobin prepared from horse blood we were also interested in following the distribution of copper in similar blood. 400 cc. of blood were drawn directly from the jugular vein of a horse into a flask containing 20 cc. of oxalate solution. Two samples of whole blood equivalent to 100 cc. of blood were centrifuged to separate the corpuscles from the serum. The serum was removed by a micro suction pipette and the corpuscles and serum were dried separately. Each sample was carefully ashed in platinum dishes which had been thoroughly cleaned, and the copper determined by the same method as was used for the hemoglobin. The average copper content for the whole blood was found to be 0.058 mg. per 100 cc. The amount of copper in the corpuscles from 100 cc. of blood was found to be

0.045 mg. No copper could be detected in either sample of serum. Another preparation of serum in which case the corpuscles were washed with isotonic NaCl solution and combined with the serum also gave a negative test for copper by our method.

Since our results were not in accord with those of Abderhalden and McHargue, we thought that perhaps the use of the oxalate in our work might cause the difference. Copper oxalate is insoluble in cold water; therefore, if the copper were in the serum it might be carried down with the corpuscles upon centrifuging the blood. To test this hypothesis a new sample of horse blood was taken, which instead of being treated with oxalate to prevent clotting, was defibrinated by shaking with glass beads. 100 cc. of defibrinated blood were dried directly, 100 cc. were centrifuged to separate the cells from the serum, and each dried separately, and an additional 100 cc. were treated in a similar manner except the corpuscles were washed with 0.9 per cent NaCl solution and added to the serum before drying. The analyses of the different preparations are given in Table I.

TABLE I.

*Copper Content of Blood Constituents.*

|  | Mg. Cu. |
|---|---|
| 100 cc. defibrinated blood... | 0.054 |
| Corpuscles from 100 cc. blood | 0.043 |
| Serum from 100 cc. blood | 0.014 |
| Washed corpuscles from 100 cc. blood | 0.032 |
| Serum + washings from 100 cc. blood | 0.017 |

The copper content of defibrinated blood agrees very well with whole blood which indicates that none of the copper is associated with the fibrin. Fibrin was also found to be copper-free by actual test. The serum from defibrinated blood contained a detectable amount of copper. Whether the difference is due to the absence of oxalate solution or to the small amount of laking which always takes place upon defibrination with glass beads, is difficult to decide. The largest amount of copper is found in the corpuscle fraction of the blood. Even after washing, the corpuscles contain the greater proportion of the copper. To establish the exact relation of these minute traces of copper and the blood necessitates further work.

### SUMMARY.

If the molecular weight of hemoglobin is accepted as 16,700 or 66,800 as recently reported by Svedberg and Fahraeus, then the hemoglobin of rat blood does not contain copper as part of its molecule.

The copper content of horse blood is approximately 0.05 mg. of Cu per 100 cc. of blood.

The corpuscle fraction of blood, whether prepared by centrifuging oxalated blood or defibrinated blood, contains the largest portion of the copper.

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# THE EFFECT OF DIET ON THE COPPER CONTENT OF MILK.*

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The recent work in this laboratory (1), demonstrating the importance of copper as a supplement to iron in the prevention of anemia in rats kept on a diet of whole cow's milk, naturally arouses interest in the copper content of milk, and in any factors affecting the amount of this element in milk.

Figures for the copper content of milk given by some of the earlier investigators (2–4) vary from 0.38 mg. to 1.4 mg. of Cu per liter. Supplee and Bellis (5) found the average of twenty-three samples of cow's milk to be 0.52 mg. per liter. These workers have not only been interested in detecting and estimating quantitatively the copper in milk, but also in studying the relation of the copper content of the milk to its flavor and vitamin potency. As early as 1905 Golding and Feilman (6) traced peculiar flavors found in market milk to equipment with exposed copper surfaces. Since then others have demonstrated similar off flavors in butter made from cream to which minute quantities of copper had been added. Hess and Unger (7) have recently shown that if milk is contaminated with copper the activity of the antiscorbutic vitamin is reduced.

The average of all the figures given in the above work is about 0.5 mg. of copper per liter. This figure appears to be too high when correlated with the results obtained in our rat feeding experiments. We have found that a definite regeneration of hemoglobin is produced by the addition of 0.005 mg. of copper and 0.5

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mg. of iron to the milk fed an anemic rat.   On the assumption
that the above figure for the copper content of milk is correct,
a rat consuming about 35 cc. of milk daily would receive 0.017 mg.
of copper per day.   The addition of 0.005 mg. of copper or less
than one-third the amount the rat is already receiving could
hardly produce such a decided effect upon the hemoglobin pro-
duction.   The presence of this relatively large amount of copper
in milk would allow regeneration of hemoglobin in a rat when fed
an iron salt alone if the milk consumption were increased by only
10 cc.

We were interested, therefore, in following the copper content
of milk to determine the actual amount present in cow's milk and
to detect any variation which might appear in milk produced un-
der different conditions.   Different workers have reported wide
variations in the time required for rats to become anemic on a
whole milk diet.   This study will determine if the copper content
of milk can be varied enough to account for these differences.   It
will also show whether the copper content of cow's milk can be
increased appreciably by the addition of copper salts to the normal
ration of a cow.   If this should prove to be true a simple means of
enhancing the nutritional value of cow's milk would be available.

In this paper we wish to present the analyses of samples of milk
from individual cows and goats fed a normal ration or one supple-
mented with copper, and the analyses of composite samples of milk
obtained from herds of cows located in various sections of the
United States.

<div align="center">EXPERIMENTAL.</div>

Three Holstein cows, receiving a standard ration of alfalfa hay,
silage, and a grain mixture were used for the copper feeding ex-
periments.   A weekly sample of milk was taken from each cow at
individual milkings.   The samples were collected immediately in
stoppered glass jars to prevent any possible contamination with
copper, and taken directly to the laboratory for analysis.   Thereby
the use of a preservative was eliminated.   These weekly analyses
were continued for a period of 4 weeks, after which copper addi-
tions were made to the rations of three cows.

The copper content of the ration was determined and the daily
intake for each cow was found to be approximately 60 mg. of cop-

per. An addition of copper equivalent to 5 times this amount was added to the ration of each cow. This amounted to 1.2 gm. of $CuSO_4 \cdot 5H_2O$ which was dissolved in water, and added to the grain mixture before each daily feeding. Samples of milk were then collected and analyzed during the 5 following weeks.

In order to enlarge upon this study samples of milk collected from various parts of the United States were analyzed for copper. Milk was collected from herds located in the following states: Maine, Vermont, New York, North Carolina, Mississippi, Texas, Missouri, Iowa, North Dakota, Colorado, Oregon, Washington, and California, representing practically every region of the country. We previously prepared the bottles used for the collection and shipping by adding a few drops of formaldehyde for the preservation of the milk. The formaldehyde used had been redistilled and found free from copper by actual test.

The samples were taken from mixed herds receiving rations which were typical of their particular section of the country. We tried to select herds which were fed only feed grown locally. In many cases, of course, the grains and the concentrates were commercial products, but in most cases the roughage was home grown. We wish to thank the men who collected the samples at the different State Experiment Stations for their kind cooperation.

Milk from goats on a normal ration and on this diet supplemented with copper was also analyzed to determine if any increase in the copper content could be detected. For this work two goats were placed in pens in our metabolism room and fed a ration consisting of 1 part of alfalfa hay and 2 parts of a grain mixture. Distilled water was supplied *ad libitum*. The grain mixture consisted of yellow corn, wheat bran, oil meal, and sodium chloride. The goats were milked twice daily, and aliquots of each milking composited for 1 week. The samples were preserved with redistilled formaldehyde. Samples were collected for a period of 3 weeks, during which time the goats received only the normal ration.

At the beginning of the 4th week 50 mg. of copper as copper sulfate were added to the ration of Goat 1 and 25 mg. of copper as copper sulfate to the ration of Goat 2. These additions increased the copper intake 10- and 5-fold respectively, since the daily copper intake due to the ration was equivalent to 5 mg.

Similarly samples were collected ¡for a period of 4 weeks.   Copper analyses were made on each of the weekly samples.

## *Method of Analysis.*

The first method used for determining the copper content of milk was a modification of the ethyl xanthate method (7).   The copper was separated from the calcium phosphate of the milk ash by precipitation with hydrogen sulfide.   A number of analyses were made by this method giving an average copper content of 0.32 mg. per liter.   In this procedure a 250 cc. sample was ignited in two 4-inch porcelain evaporating dishes and combined as a single sample after ignition.   No attention was given in these analyses to the possible presence of copper in the porcelain dishes, but upon igniting a 1 gm. sample of sodium acetate in a new porcelain dish as much as 0.1 mg. of copper was extracted.   If two new dishes were used an increase of 0.2 mg. per sample would be obtained and as much as 0.8 mg. of copper per liter would be reported in the milk which actually came from the dish.   This is one step in the procedure which must be closely guarded or too high results will be obtained.   The purity of the reagents, especially the distilled water, must also be watched carefully.

All the analyses reported in this paper were made on samples ignited in quartz (vitreosil) dishes.   We found that quartz dishes contained much less copper than the porcelain ones.   Quartz dishes very seldom gave a test for copper when ignited with sodium acetate; however, they were always thoroughly extracted before being used.   Several samples were also ashed in platinum dishes but since the results obtained when platinum was used checked very well with those obtained when the quartz dishes were used, ignition of samples in vitreosil dishes was resorted to entirely.

The milk ash was taken up in 10 cc. of 1:1 HCl, filtered, and diluted to 200 cc.   The solution was warmed, and washed $H_2S$ passed in for 15 minutes.   The solution was kept in a stoppered flask until the precipitate of CuS settled out.   The copper sulfide was then filtered off, oxidized with nitric acid, and the amount of copper determined in exactly the same manner as outlined by Elvehjem and Lindow (8).   The amount of copper found in 250 cc. was multiplied by four to obtain the amount per liter.

The results of the analysis of milk from three cows when

fed a normal ration and when fed a normal ration to which $CuSO_4.5H_2O$ equivalent to 5 times the copper content of the ration was added, are given in Table I.

The copper content of the milk produced by cows on a normal ration averages about 0.15 mg. per liter. This figure is considerably lower than most of the figures for raw milk reported in the literature. We believe that many of the high figures reported are due to copper contamination during the process of analysis,

TABLE I.

*Copper Content of Milk from Cows before and after Receiving Copper Additions.*

| Date. | Mg. Cu per liter milk. | | |
|---|---|---|---|
| | Cow 21. | Cow 22. | Cow 23. |
| *1928* | | | |
| Apr. 24 | 0.157 | 0.155 | 0.197 |
| May 18 | 0.151 | 0.155 | 0.128 |
| " 24 | 0.127 | 0.123 | 0.175 |
| " 28 | 0 182 | 0.155 | 0.124 |
| Average............ | 0.154 | 0.147 | 0.156 |
| June 6 | Cu added to ration. | | |
| June 13 | 0.196 | 0.145 | 0.139 |
| " 20 | 0.124 | 0.136 | 0.152 |
| " 27 | 0.172 | 0.120 | 0.147 |
| July 11 | 0.123 | 0.149 | 0.133 |
| Average............ | 0.154 | 0.137 | 0 143 |

especially from the dishes used for the ignition of the milk. The recent work of Quam and Hellwig (9) also places the figure at about 0.4 mg. per liter. They state that the possibility of copper contamination during the analysis was checked but they did not state that the possibility of contamination during the ashing process was investigated.

The figures for the milk from the same cows when on a ration containing a 5-fold increase in copper are no higher than those for the normal milk. This demonstrates definitely that the copper content of milk cannot be influenced by increasing the copper intake within reasonable limits.

In Table II are tabulated the analyses of the milks obtained from the different sections of the United States   These results are of the same magnitude as those given in Table I.   The figures

TABLE II

*Copper Content of Milk Produced in Different Sections of the United States*

| State | Cu content | State | Cu content |
|-------|-----------|-------|-----------|
|  | *mg  per l* |  | *mg  per l* |
| Maine | 0 168 | Iowa | 0 150 |
| Vermont | 0 137 | North Dakota | 0 174 |
| New York | 0 158 | Colorado | 0 130 |
| North Carolina | 0 123 | Oregon | 0 129 |
| Mississippi | 0 143 | Washington | 0 136 |
| Texas | 0 184 | California | 0 142 |
| Missouri | 0 136 |  |  |

TABLE III

*Copper Content of Milk from Goats before and after Supplementing the Ration with Copper*

| Wks  on experiment | Goat I | Goat II |
|-------------------|--------|---------|
|  | *mg  Cu per l* | *mg  Cu per l* |
| 1 | 0 121 | 0 164 |
| 2 | 0 142 | 0 127 |
| 3 | 0 196 | 0 140 |
| 4 | 0 167 | 0 116 |
| Average | 0 156 | 0 137 |
|  | 50 mg   Cu added to ration daily | 25 mg   Cu added to ration daily |
| 5 | 0 141 | 0 142 |
| 6 | 0 162 | 0 125 |
| 7 | 0 164 | 0 114 |
| 8 | 0 153 | 0 140 |
| Average | 0 155 | 0 130 |

range from 0.123 mg. per liter for the milk from North Carolina to 0.184 mg. per liter for the milk from Texas.   The variations are so slight that no significance can be attached to the differences. These figures demonstrate that the copper content of cow's milk

is practically the same even if produced under varied conditions. The difference in the rate of anemia production in rats on whole milk diets reported by different investigators, therefore, cannot be due to a variation in the copper content of the milk when produced, but rather due to the contamination of the milk after production or to unknown sources of copper supply during the different periods of the rat's life.

The amount of copper found in these samples of milk also substantiates our biological findings.    If a rat consumes 35 cc. of milk containing 0.15 mg. of copper per liter, the daily copper intake would be 0.005 mg.    The addition of 0.005 mg. of copper (the amount necessary for hemoglobin regeneration in a rat) would double the copper intake.    This would be a great enough change in the intake to account for the rapid stimulation in the hemoglobin production.

The results obtained by the analysis of goat milk are given in Table III.    The copper content of goat milk is almost the same as that of cow's milk.    We did not find the figure for goat milk decidedly lower than that for cow's milk as was reported by Quam and Hellwig (9).    The addition of 50 or 25 mg. of copper daily to the ration of the goat did not influence the copper content of the milk.

### SUMMARY.

1. Milk produced by cows on a normal ration contains about 0.15 mg. of copper per liter.

2. The copper content of cow's milk cannot be increased by feeding the cows sufficient copper sulfate to increase the copper intake 5-fold.

3. Samples of cow's milk collected from thirteen herds located in different states showed very slight differences in copper content.

4. Increasing the copper content of the ration 5- to 10-fold did not increase the copper content of goat milk.    Further, limited numbers of analyses for copper did not indicate a decidedly lower amount of this element in goat milk as compared with cow's milk.

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# THE QUANTITATIVE DETERMINATION OF BILE ACIDS BY MEANS OF A NEW COLOR REACTION AND MONOCHROMATIC LIGHT.

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A survey of the literature reveals the fact that numerous methods, direct and indirect, have been employed by various workers for the determination of bile acids. Due to the many advantages offered by a colorimetric method for the determination of small quantities of a substance, the Pettenkofer reaction (1) as modified by Mylius (2) seemed most worthy of consideration. A critical study of the quantitative possibilities of this reaction, however, soon revealed several undesirable features: (1) The *red* or *reddish violet* color varied greatly as to color shade, making comparison in the colorimeter very difficult; (2) the quantitative recovery of pure sodium glycocholate was very irregular with errors of from 1 to 12 per cent; (3) the color reaction was not specific for bile acids, Mylius finding 150 other substances which gave the Pettenkofer reaction. It was obvious that the Pettenkofer reaction, as commonly described, was wholly unsuitable for quantitative purposes even on pure solutions of bile acids and unreliable for even qualitative work on body fluids.

During the course of a spectrophotometric study of the reaction between glycocholic and sulfuric acids and furfural, a blue-colored compound was discovered. When to a dilute bile acid solution were added 34 volume per cent of $H_2SO_4$ and 0.05 volume per cent of furfural and the mixture heated for 30 minutes at 65°, a pure *blue* color results. This color is different from any that has been reported in the literature. Pettenkofer (1) described the color he obtained as violet-red, Vogel and Dragendorff (3) obtained a brown color, Mylius (2) describes the end-product as "blood red," Jolles (4) and Hirschfelder (5) reported a red to brown color, Gilbert,

Chabrol, and Bénard (6) obtained red to red-purple, Herzfeld and Haemmerli (7) report a variety of colors from faint green to blue-green, and Roundtree, Greene, and Aldrich (8) reported a pink color.

The blue-colored compound was found to be stable for 2 to 3 hours, perfectly reproducible and quantitative in nature; *i.e.*, conforms to Beer's law. The latter was determined by calculating the constant of deviation from Beer's law by the formula of Kober, as given by Yoe (9). Negligible deviations were obtained in all cases. In order to distinguish this reaction giving a blue color it is called the Gregory reaction.

### EXPERIMENTAL.

Glycocholic acid was purified by recrystallization from alcohol by the addition of ether and a little HCl, and furfural by repeated distillations. Portions of sodium glycocholate solutions of known concentration (0.1 and 0.2 mg. per cc.) were measured into test-tubes with a calibrated micro burette. The volume was then made to 1 cc. with distilled water, 6 cc. of 45 per cent $H_2SO_4$ were added, and finally 1 cc. of 0.3 per cent furfural solution. The tubes were loosely stoppered and set in a water bath at 65° for 30 minutes. The results are shown in Table I. It is noteworthy that in all cases when the concentration was less than that of the standard the error is positive in sign while a greater concentration than the standard always gave a negative error.

Various substances other than bile acids that give a positive Pettenkofer reaction were subjected to the Gregory reaction and found to be negative. These include glycine, taurine, cholesterol, lanoline, lecithin, cephalin, and oleic acid. Since these include the substances that might be present in blood and that give a positive Pettenkofer test and since it is shown below that an alcoholic extract of normal blood does not give the Gregory reaction, the latter is adequate for the determination of bile acids in an alcoholic extract of blood.

### *Experiments on Dog Bile.*

5 cc. of a sodium glycocholate solution containing 1 mg. per cc. were treated for bile with animal charcoal and kaolin according to the procedure of McClure, Vance, and Greene (10). The Gregory

reaction was tried on aliquots of the filtrate corresponding to $\frac{1}{3}$ and $\frac{2}{3}$ mg. and compared with a standard containing $\frac{1}{2}$ mg. of sodium glycocholate. In the first tube no color developed. The second tube was found to contain only 0.5 mg. of sodium glycocholate

TABLE I.

*Results from Analyses of Solutions of Pure Sodium Glycocholate with the Gregory Procedure.*

| Series No. | Sodium glycocholate, theoretical. | Found. | Error. |
|:---:|:---:|:---:|:---:|
| | *mg.* | *mg.* | *per cent* |
| 1 | 0.8 | 0.796 | −0.75 |
| | 0.9 | 0.830 | −0.78 |
| | 1.0 | | Standard. |
| | 1 1 | 1.075 | −2.20 |
| | 1 2 | 1.176 | −2.00 |
| 2 | 1.0 | | Standard. |
| | 0.9 | 0.909 | +1 00 |
| | 0.8 | 0.813 | +1.60 |
| | 0.7 | 0.720 | +2.85 |
| | 0.6 | 0.631 | +5.17 |
| 3 | 0.6 | 0.623 | +3.71 |
| | 0.8 | 0.810 | +1.25 |
| | 1 0 | | Standard. |
| | 1.2 | 1.170 | −2.50 |
| | 1.4 | 1.360 | −2.85 |
| 4 | 1.0 | | Standard. |
| | 1.2 | 1.185 | −1.25 |
| | 1.4 | 1.350 | −3.57 |
| | 1.6 | 1.460 | −8.75 |
| | 1.8 | 1.680 | −6.67 |
| | 2.0 | 1.800 | −10.00 |

although the theoretical concentration was $\frac{2}{3}$ mg. From this experiment it is quite evident that adsorption on the charcoal and kaolin removed approximately 80 per cent of the bile salt present in the original solution, and hence their method of removing bile pigments cannot be used.

Bile was extracted with alcohol according to Foster and Hooper

2.95 mm. thick, medium shade.   This monochromatic light was used to illuminate a Bausch and Lomb colorimeter.

10 cc. of dog fistula bile were treated with 8 volumes of 95 per cent ethyl alcohol.   The mixture was brought to boiling, filtered, and the filtrate was diluted with alcohol to a volume of 250 cc. 1 cc. portions of the filtrate were evaporated to dryness in test-tubes and known quantities of sodium glycocholate were added. The results showing the recovery of the added sodium glycocholate after treatment according to the Gregory procedure are shown in Table II.

In no instance did the presence of the relatively large amounts of bile pigment interfere with the comparison of the color in the bile solutions with standards prepared from pure sodium glycocholate.

Another series of determinations was made upon a different sample of bile, treated as before and finally diluted 1:10.   The results are shown in Table III.

All of the final solutions in the second series were markedly colored owing to the quantity of bile pigments present.   Color comparisons with the colorimeter illuminated with daylight were impossible, yet by use of the monochromatic light no difficulties were encountered.

### Tests for Bile Acids in Normal Blood.

5 liters of fresh ox blood were treated with 3 volumes of 95 per cent ethyl alcohol, brought to boiling, and filtered.   The coagulum was twice extracted with 1 volume of hot alcohol.   The combined filtrates were concentrated *in vacuo* to small volume and the residue was treated with 4 volumes of 95 per cent ethyl alcohol and filtered.   After evaporation to dryness the residue was taken up in 200 cc. of hot water.   After cooling, solid $MgSO_4$ was then added to complete saturation and the solution was centrifuged. The fatty layer was removed, extracted with 50 per cent alcohol, and made up to a final volume of 100 cc.   The fat-free $MgSO_4$ solution was made up to a volume of 200 cc., 25 cc. aliquots were removed, evaporated to dryness, extracted with alcohol, and the alcoholic extracts diluted to 50 cc.   2 cc. portions of the extract from the fatty material and of the alcoholic solution of fat-free

material, when treated according to Gregory's procedure and with the special illumination, failed to show the presence of bile acids. These results confirmed those of a previous attempt by one of us (R.G.) to demonstrate the presence of bile acids in normal blood. It should be noted that in the foregoing procedures two common sources of errors were avoided. First, no clarifying agents which are known to adsorb and remove bile acids were employed, and secondly, the blood residue was not extracted with fat solvents to remove the blood fat. Regarding the latter point, preliminary trials showed that although the bile acids or their salts are insoluble in ordinary fat solvents, they are readily soluble in the solution of fat in these solvents.

The results given here are entirely contrary to the results recently published by Roundtree, Greene, and Aldrich (8). These investigators, using a modified Pettenkofer procedure, report the presence of 2.5 to 6 mg. of bile salts per 100 cc. of normal human blood. Their method does not exclude cholesterol and fatty acids which gave a positive Pettenkofer test.

### SUMMARY.

1. The Pettenkofer reaction was studied and found unsuitable for the quantitative estimation of bile acids in pure solutions or body fluids.

2. A new color reaction for the determination of bile acids is described which is more specific and more accurate than the Pettenkofer procedure.

3. A new and simple apparatus for monochromatic illumination of the colorimeter was devised for use in the study of the bile acid content of bile and blood.

4. By means of the new color reaction and monochromatic light it was found possible to determine bile salts in bile quantitatively, which is not possible with the Pettenkofer reaction.

5. No bile salts were found in two trials with about 5 liters of blood each.

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# DETERMINATION OF CARBON MONOXIDE IN BLOOD.

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Methods suggested for the determination of carbon monoxide
in blood may be considered as belonging to either of these two
classes: first, those in which the relative content of carbon mon-
oxide in blood is measured; second, those in which the absolute
quantity of carbon monoxide per given quantity of blood is
determined.

In the first class, the result is expressed as the percentage
saturation of hemoglobin with carbon monoxide.   Usually a few
drops of blood suffice for a determination.   The principal methods
belonging to this class are: (*a*) Haldane's carmine method (1),
whereby the carbon monoxide content is determined from the
color of the greatly diluted blood solution, which is more or less
pink, depending on the percentage saturation of the hemoglobin
with carbon monoxide.   This method is not very exact, its results
being largely dependent upon subjective causes.   (*b*) Sayers and
Yant's pyrotannic acid method (2).   This is based on the fact that
after the addition of tannic acid to normal diluted blood a gray
color is produced, whereas under the same conditions, blood
containing carbon monoxide remains more or less red.   The per-
centage saturation is found by comparison with a color scale.   This
method is open to the same criticisms as Haldane's carmine
method.   (*c*) Spectroscopic determination.   Here the percentage
saturation of hemoglobin with carbon monoxide is found by the
amount of displacement of the absorption bands of the blood,
which are displaced more towards the violet end of the scale,
depending upon the amount of carbon monoxide present.   Hart-
ridge's reversion spectroscope (3) is well suited to this method,
but is very expensive.

In the methods belonging to the second class, the result is

expressed in cc. of carbon monoxide (at $0°$ and 760 mm.) per cc.
of blood. For such determinations, several cc. of blood are gener-
ally required. By these methods, the carbon monoxide is deter-
mined after its liberation. The principal methods belonging to
this class may be briefly discussed: (*a*) Nicloux (4) liberates the
carbon monoxide through addition of phosphoric acid, after which
the gas is determined eudiometrically This procedure is not
very accurate in the case of small quantities. (*b*) Van Slyke has
proposed several methods, in which the carbon monoxide is freed
by means of ferricyanide and determined gasometrically. In



Fig. 1.

the improved procedure of Van Slyke and Robscheit-Robbins (5),
the error is reduced to 0.02 to 0.03 volumes per cent. The amount
of blood recommended for a determination here is 5 cc. (*c*) Cohen
Tervaert (6) liberates the carbon monoxide by the addition of
potassium ferricyanide in a vacuum. The carbon monoxide then
reacts with iodine pentoxide heated to $150°$, the following reaction
taking place: $I_2O_5 + 5CO \rightarrow 5CO_2 + I_2$. The iodine freed is
absorbed in a potassium iodide solution and titrated with 0.01 N
thiosulfate solution from a micro burette.

The Cohen Tervaert procedure is subject to the following criti-
cisms. The amount of blood necessary for a determination is
10 cc., necessitating venepuncture. The large Peligot tubes of

the apparatus seem unsuitable, especially for taking up the iodine; some of the iodine is absorbed by the rubber stoppers and tubing. The concentration of the potassium iodide solution recommended (0.5 per cent) is too low, and this results in low figures in cases of high carbon monoxide content.

These drawbacks in this method have led me to modify it and at the same time convert it into a micro method. Only 1 cc. of blood, which can be obtained from a finger tip or ear lobe, is necessary for a determination. The modified apparatus is shown in Fig. 1.

This figure shows that the gas mixture under analysis is contained in a 100 cc. bottle, closed with a 3-hole rubber stopper.
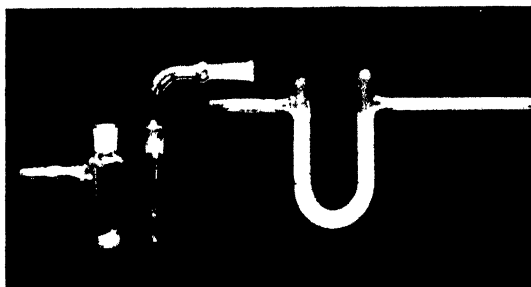


FIG. 2.

Through one of these holes, there passes a small glass funnel with a stop-cock; through the other two holes there pass two capillary tubes (bent at right angles) connected with pressure tubing equipped with screw clamps. When the gas mixture is suctioned off through the apparatus (by means of a Mariotte bottle), it passes successively from right to left as follows: through a safety capillary tube (which prevents water from being suctioned into the apparatus), an absorption tube filled with soda-lime and calcium chloride, another such tube filled with phosphorus pentoxide, the U-tube with iodine pentoxide (which is kept at 150° in an air bath), and finally through the absorption bottle filled with a potassium iodide solution, in which the iodine dissolves.

The connections between the U-tube and the absorption bottle are of ground glass, as Fig. 2 shows. No rubber connections

should be used here, nor should the ground glass connections be greased. The iodine pentoxide is made more reactive by mixing it with a little very short glass wool. At each end of the glass tube, glass wool is also inserted. For closing this tube the glass is melted  The U-tube contains about 8 gm. of iodine pentoxide which is sufficient for a number of determinations.

### Method.

The stoppered bottle is evacuated and the screw clamps tightly shut. 1 cc. of blood is cautiously introduced into the bottle by way of the small glass funnel and stop-cock. Similarly 1.5 to 2 cc. of distilled water are introduced and this is mixed with the blood till hemolysis is complete. 1 cc. of a freshly prepared, cold saturated solution of potassium ferricyanide is now added and the mixture is well shaken. The bottle is then kept in a water bath at 40° for about half an hour, being shaken vigorously at intervals. One of the clamps is now opened to permit the introduction of pure air into the bottle. Then one of the pieces of pressure tubing is connected with the elbow-shaped tube which stands in a large glass beaker filled with water, and the other length of pressure tubing is connected with the capillary safety tube, after which the gas mixture is lead into the apparatus. This may take 10 to 15 minutes. (The small absorption bottle contains about 1 gm. of potassium iodide dissolved in 1 cc. of distilled water.) Finally about 150 to 300 cc. of pure air are passed through the apparatus. The absorption bottle is now removed and the iodine in it titrated with 0.001 N thiosulfate solution from a micro burette, a few drops of starch solution being added towards the end of the titration. 1 cc. of 0.001 N thiosulfate solution is equivalent to 0.056 cc. of carbon monoxide at 0° and 760 mm.

Before the apparatus is used, pure air at a temperature of 200 to 230° should be passed over the iodine pentoxide until at 150° practically no more iodine is evolved.[1]  As iodine pentoxide is

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[1] For very accurate work, a blank should be run. On the other hand, if very pure iodine pentoxide is used, one has, in most cases, to add a blank value. This is due to the fact, that, in order to reach the end-point with starch, a definite minimum amount of iodine is necessary, which is not given off in the blank test. In such cases one titrates in the blank test with 0.001 N iodine solution until the end-point is reached. The value so found is added to that found in the unknown determinations. For very accurate work it is also essential that the volumes of the unknown and blank shall be the same at the end of the titration.

very hygroscopic, the U-tube should always be closed off from the open air. It is important that the titration be carried out slowly towards the end, as the speed of reaction is very slow with solutions that are so dilute. The 0.001 N thiosulfate solution should be freshly prepared each day before use by dilution of a 0.1 N stock solution with boiled, distilled water. When the apparatus has not been used for some time, pure air at 150° should

TABLE I.

*Recovery of Carbon Monoxide from Air.*

The results are expressed as cc of CO (at 0° and 760 mm.) per liter of air.

| Sample No. | CO calculated | CO found. | Sample No | CO calculated. | CO found. |
|---|---|---|---|---|---|
| 1 | 0 98 | 0 99 | 4 | 1 93 | 1 88 |
| | | 1 01 | | | 1 93 |
| | | 0 96 | | | 1 89 |
| | | 0 97 | | | 1 91 |
| | | 0 98 | | | 1 86 |
| 2 | 0 58 | 0 57 | | | 1.87 |
| | | 0 58 | | | 1 89 |
| | | 0 56 | 5 | 0 19 | 0 21 |
| | | 0 57 | | | 0 21 |
| | | 0 57 | | | 0 20 |
| | | 0 56 | | | 0 20 |
| | | 0 57 | | | 0 19 |
| 3 | 0 20 | 0 23 | | | 0 20 |
| | | 0 21 | 6 | 0 47 | 0 47 |
| | | 0 19 | | | 0 51 |
| | | 0 21 | | | 0 49 |
| | | 0 21 | | | 0 47 |
| | | | | | 0 48 |

first be passed through it. The absorption tubes, chiefly the one with calcium chloride and soda-lime, should be renewed frequently. Whenever old tubes are used there is some escape of iodine, even when the gas transferred contains no carbon monoxide.

## Results.

Table I gives the results of analysing 100 cc. of gas mixtures prepared by mixing 8 liters of pure air with known quantities of carbon monoxide.

The method was next applied to blood (defibrinated calf blood). The results are given in Table II.

As the method is used, the blood is taken from a finger tip and introduced into a small tube (graduated to indicate 1 cc. of blood) containing 0.5 cc. of a 1 per cent sodium citrate solution. When citrate is used it is advisable to employ more water (about 3 cc.)

TABLE II.

*Recovery of Carbon Monoxide from Blood.*

The results are expressed as cc. of CO (at 0° and 760 mm.) per cc. of blood.

| | Quantity of Sample 1. | Quantity of Sample 2. | CO calculated. | CO found. | Average. |
|---|---|---|---|---|---|
| | cc. | cc. | | | |
| Sample 1. | | | | 0.000 | |
| | | | | 0.003 | |
| | | | | 0.001 | 0.001 |
| Sample 2. | | | | 0.176 | |
| | | | | 0.174 | |
| | | | | 0.174 | 0.175 |
| Mixture 1. | 20 | 5 | 0 036 | 0.040 | |
| | | | | 0.035 | |
| | | | | 0.035 | 0.037 |
| Mixture 2. | 20 | 10 | 0.059 | 0.061 | |
| | | | | 0.057 | |
| | | | | 0.056 | 0.058 |
| Mixture 3. | 10 | 10 | 0.088 | 0.090 | |
| | | | | 0.091 | |
| | | | | 0 086 | 0.089 |
| Mixture 4. | 10 | 20 | 0.117 | 0.120 | |
| | | | | 0.115 | |
| | | | | 0.114 | 0.116 |

for the hemolysis. The following figures show that the use of the anticoagulant has no influence on the results. A sample of blood without any citrate added showed a carbon monoxide content of 0.084 cc. per cc. of blood; the same sample with citrate added gave a figure of 0.085 cc. per cc. of blood (average of three determinations).

The method described in this paper is being applied in the

Laboratory of Technical Hygiene of the examination of blood. from people who, as a result of their occupation, are in frequent

TABLE III.

*Carbon Monoxide Content of Blood of Persons Exposed to Carbon Monoxide Fumes.*

| Experiment No. | CO per cc. blood. | Remarks. |
|---|---|---|
| | *cc* | |
| 1 | 0 006 | Chauffeur A. Open Ford car driven 6 hrs. with exhaust gas escaping from motor. Blood taken immediately on leaving car. |
| 2 | 0 005 | Female passenger in same car. Blood taken after ½ hr. walk. |
| 3 | 0 006 | Another chauffeur, B, in same car on another day. Blood taken after 4½ hrs. service. |
| 4 | 0 014 | Male passenger in same car. Blood taken on leaving car. |
| 5 | 0 000 | Blood taken before riding in car |
| 6 | 0 004 | Blood from same individual as in Experiment 5, taken after short ride in same car. |
| 7 | 0 006 | Mechanic C. Practically no engine had turned in garage; doors kept open. |
| 8 | 0 005 | Mechanic D. Same garage and conditions as in Experiment 7 |
| 9 | 0 012 | Mechanic C Garage doors kept open. An engine had run occasionally. |
| 10 | 0 007 | Mechanic E. Same conditions as in Experiment 9. |
| 11 | 0 014 | Chauffeur F. Conditions practically same as in Experiment 9. |
| 12 | 0 018 | Mechanic D in same garage. Doors had been shut; an engine had turned occasionally. |
| 13 | 0 018 | Mechanic E Same conditions as in Experiment 12. |
| 14 | 0 015 | Chauffeur F. Blood taken after 4 hrs. service on his car and some time in garage. |
| 15 | 0 006 | Omnibus Chauffeur G. Blood taken after 8 hrs. service. |
| 16 | 0.001 | " " H. " " " 6¼ " " |
| 17 | 0 003 | " " I. " " " 7½ " " |
| 18 | 0 007 | " " J. " " " 7½ " " |
| 19 | 0 000 | " " K. " " " ½ hr. " |

contact with carbon monoxide. Motor car drivers and garage people are those most frequently exposed.[2] The results in a number of cases are given in Table III.

[2] An examination of the air in garages, as also in motor cars, according to Hahn and Hirsch's method (7), has already been made. The results will soon be given in *Zeitschrift für Hygiene und Infektionskrankheiten.*

The hemoglobin content (Sahli) was determined in the last fifteen cases of Table III. It was normal (85 to 95 per cent) in all cases except in Experiment 16, high (105 per cent), Experiments 17 and 19, low (72 and 73 per cent respectively). Mechanics A and B complained about their health, suffering from headache, etc. Both appeared very pale.

It may be stated that 1 cc. of blood from an adult with an average hemoglobin content can contain a maximum of about 0.250 cc. of carbon monoxide. Whenever 20 to 30 per cent of this is present, rather serious acute symptoms can be found. Almost all the cases in Table III had a little carbon monoxide in their blood, which, while not enough to account for acute symptoms, ought not to be disregarded if one is regularly exposed to carbon monoxide.

### SUMMARY.

1. A modification of Cohen Tervaert's method for the quantitative estimation of carbon monoxide in blood, in which only 1 cc. of blood is required, is described.

2. The carbon monoxide content of the blood of a number of cases exposed to carbon monoxide is given.

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# GASOMETRIC DETERMINATION OF FERMENTABLE SUGAR IN BLOOD AND URINE.

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## INTRODUCTION.

The method previously described by the authors (1928) for gasometric determination of total reducing substances in blood , and urine is in this paper applied to the estimation of fermentable sugar in both fluids.

Approximate determination of fermentable sugar in urine by manometric determination of the $CO_2$ formed by yeast is also described.

Procedures for fermentative determination, in blood and urine, of glucose, or of sugars resembling it in their fermentability by yeast, have been refined by different investigators during the past few years. The chief source of error, undue prolongation of the time of fermentation to one or more days, was removed by Hiller, Linder, and Van Slyke (1925), who utilized conditions under which glucose could be completely removed from blood in a half hour. Folin and Svedberg (1926), Somogyi (1927, 1928), Benedict (1928), and Raymond and Blanco (1928) have utilized these conditions for blood analyses, and Eagle (1926–27) for urine. All the above authors estimated fermentable sugar by the decrease in reducing substances caused by fermentation with yeast. To the technique Somogyi added the improvement of using washed yeast cells, so that correction for reducing substances adherent to the yeast could be obviated. Lund and Wolfe (1926) used a short fermentation period for urine, with a pure culture of yeast, which permitted them to determine with a Barcroft apparatus the $CO_2$ formed as a measure of the sugar fermented.

By all these authors except Lund and Wolfe the reducing sugars have been estimated by difference, according to the equation:

$$(1) \qquad F = T - N$$

where $F$ = fermentable sugar, $T$ = total reducing substances, and $N$ = non-fermentable reducing substances.

A factor of minor but measurable influence on the precision of results in most of the procedures used is the permeability of the yeast cells to the non-fermentable reducing substances of blood and urine. The existence or non-existence of such permeability must be considered in calculating results when the latter are based on analyses of aliquot parts of the yeast-blood or yeast-urine filtrate. Hiller, Linder, and Van Slyke (1925) avoided the necessity of considering this factor, because they used the Hagedorn-Jensen (1918, 1923) blood sugar method. This method is unique in that the coagulated blood protein precipitate is quantitatively extracted with several portions of hot water, so that the filtrate analyzed represents the entire blood sample, not an aliquot part. Some authors (Eagle, 1926-27; Folin and Svedberg, 1926; Somogyi, 1927; Benedict, 1928; Raymond and Blanco, 1928) have diluted blood or urine plus yeast to a measured volume, and analyzed an aliquot part of the filtrate, with the apparent assumption that the non-fermentable reducing substances diffused through the yeast in the same concentration as through the solution in which the cells were suspended. According to the results of Somogyi (1928), however, which we have confirmed, the non-fermentable reducing substances of blood do not diffuse into the yeast. These substances therefore are more concentrated in the filtrate of the blood + yeast mixture than in the filtrate of blood that has been diluted to the same volume without yeast. The fermentable sugar calculated by difference is diminished by the error. In the technique of Raymond and Blanco (1928) and the earlier procedure of Somogyi (1927) the yeast cells constituted 6 and 7 volumes per cent of the final mixture respectively, and their bulk introduced a corresponding positive percentage error into the $N$ of Equation 1. The effect on the value found for $F$, the fermentable sugar, caused by a 6 per cent error in $N$ is about 2 per cent of $F$, if, as in normal blood, $N$ is about one-fourth of $T$.

In urine we have found that yeast, although not entirely impermeable to the non-fermentable substances, absorbs them in relatively small amounts. The amount taken up per cc of yeast cells averages only about one-fifth the amount left in solution per cc. of fluid. We have governed accordingly our technique, outlined below. Eagle (1926-27) made no correction for the volume occupied by yeast cells (about 6 per cent of the volume of the yeast-urine mixture) in his urine fermentations. Such correction would increase his nearly negative fermentable sugar values, but in no case apparently to as high as 0.01 per cent.

### DETERMINATION OF FERMENTABLE SUGAR IN BLOOD FROM DECREASE IN REDUCING POWER CAUSED BY YEAST TREATMENT.

*Removal of Blood Proteins.*—The blood proteins are removed with the modification of Folin and Wu's (1919) tungstic acid procedure described on p. 741 of Van Slyke and Hawkins (1928).

*Removal of Fermentable Sugar from Blood Filtrate.*[1]—From one portion, conveniently about 10 cc., of the filtrate the fermentable sugar is removed according to Somogyi (1928). We have applied the procedure as follows:

A portion of Fleischmann's yeast cake is pulverized and suspended in 4 times its weight of water. Of the suspension a volume, approximately equal to that of the blood filtrate sample to be fermented, is placed in a centrifuge tube and washed five times by repeated centrifugation and decantation. After the last centrifugation the water is decanted as completely as possible, and the water film adherent to the walls of the tube above the packed cells is removed with a roll of filter paper. Adherent water remaining between the cells is not sufficient to dilute significantly the blood filtrate. The blood filtrate is added to the yeast packed in the centrifuge tube. Filtrate and yeast are mixed and permitted to stand at room temperature for 15 minutes. The mixture is then centrifuged.

*Determination of Total and Unfermentable Blood Sugar.*—3 cc. portions of the supernatant fluid obtained by the above yeast treatment and 3 cc. portions of untreated filtrate are analyzed as described by Van Slyke and Hawkins (1928) for blood sugar.

The $p_0$ value, for the analyses of the yeast-treated portions, is determined by blank analysis of the supernatant fluid obtained from a centrifuged mixture of 1 volume of washed yeast cells and 4 volumes of water.

[1] Folin and Svedberg (1926) have shown that yeast acts well in Folin-Wu blood filtrates. Somogyi states furthermore that fermentation of the filtrate instead of whole blood obviates one source of occasional error. He found that some pathological bloods when mixed with yeast reacted with it to *produce* reducing substances even in the few minutes required for the short fermentation technique. These substances in part replaced the glucose removed by the yeast, and caused erroneously low values to be calculated for fermentable sugar. Such error was obviated when the yeast acted on the blood filtrate instead of the blood itself.

Until these observations of Somogyi (1928) appeared, we had used yeast treatment of the whole blood to determine fermentable sugar, since the technique involves one filtration less than that for fermentation of the filtrate. In our analyses, limited chiefly to normal and nephritic subjects, we did not encounter any pathological blood specimens of the type mentioned by Somogyi (1928). Nevertheless in view of his experience with such specimens it appears desirable to abandon treatment of whole blood with yeast, and to treat only the blood filtrate.

It is essential in determining this blank to treat the yeast with water rather than with tungstic acid solution. When water is mixed with washed yeast we have found that it extracts the same minimal amount, if any, of reducing material from the cells that a sample of glucose-free (previously fermented) Folin-Wu blood filtrate extracts from them. Hence it appears that the water extract gives the correct blank. If yeast is mixed with tungstic acid solution, the latter extracts measurably more reducing material, enough to be equivalent to 5 or 10 mg. per cent of blood sugar,—sometimes more. Presumably the reason for this phenomenon is that filtrate from tungstic acid-yeast mixture has a much greater acidity and contains more titratable acid than filtrate from tungstic acid-blood mixture; in the latter case the blood proteins remove the acid almost completely. The effect of using a tungstic acid extract of yeast cells for the blank determination would be to lower erroneously the $p_0$ value, and hence to lower the value, calculated as $(p_0 - p_1) \times$ factor, obtained for non-fermentable reducing material in blood.

### *Calculation of Fermentable Blood Sugar.*[2]

For calculating both total sugar from analysis of the untreated filtrate, and unfermentable sugar from analysis of the yeast-treated filtrate, the $p_0 - p_1$ values are multiplied by the usual calculation factors in Table II of our former paper (1928).

If a temperature change occurs during the interval between the $p_0$ and $p_1$ observations, $p_0$ is to be corrected as indicated in their Table I.

From the total and unfermentable reducing material the fermentable sugar is found by difference.

Fermentable sugar = (total sugar) − (unfermentable sugar)

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[2] If *only* fermentable sugar is desired, only the $p_1$ values of the two analyses need be determined. Fermentable sugar is then calculated as $(p_{1T} - p_{1N}) \times$ *factor*, where $p_{1T}$ is the $p_1$ reading for the total sugar determination, $p_{1N}$ is the $p_1$ for the non-fermentable sugar analysis, and the factor is from Table II of our former paper (1928). This procedure eliminates the two $p_0$ determinations. It can be used, however, only if the yeast employed has been washed quite free of reducing substances.

## DETERMINATION OF FERMENTABLE SUGAR IN BLOOD FROM DECREASE IN REDUCING POWER CAUSED BY SPONTANEOUS GLYCOLYSIS.

Spontaneous disappearance of reducing sugar from blood was a phenomenon known to Claude Bernard and studied by many later investigators (see Tolstoi, 1924). The sugar is transformed into lactic acid, as shown by Evans (1922). Hiller, Linder, and Van Slyke (1925) and Folin and Svedberg (1926) have found that the same amount of reducing substance is removed by spontaneous glycolysis at 38° for 20 or more hours that is removable by short fermentation with yeast.

*Procedure.*—Two analyses, designated as *A* and *B* are required.

*A.*—In one sample of fresh blood the total reducing material is determined at once.

*B.*—Another sample of the whole blood in a stoppered tube is incubated at 38° for 20 to 24 hours, and the non-glucose reducing material left in it is then determined.

*Calculation.* $A - B$ = fermentable sugar.

## DETERMINATION OF FERMENTABLE SUGAR IN URINE BY MEASURING DECREASE IN REDUCING SUBSTANCES CAUSED BY YEAST TREATMENT.

The procedure outlined below, with minimum dilution of the urine, is designed primarily for urine with amounts of reducing substances of the order of magnitude found in non-diabetic cases. It is usually in urines of slight reducing power that one needs to determine the fermentability of the material, either for diagnostic or experimental purposes. The method as given is designed for urines with reducing powers not exceeding that of a 0.5 per cent glucose solution.

To determine the fermentable sugar in urines more heavily loaded with reducing substances, such urines are diluted sufficiently to bring the total reducing power below that of a 0.5 per cent glucose solution.

### Reagents for Urine Analysis.

*Ferricyanide Solution.*—This contains 14 grams of $K_3Fe(CN)_6$, 75 grams of $K_2CO_3$, and 75 grams of $KHCO_3$ per liter. It is identical with the reagent described for urine in our previous paper (1928), except that here only half as much ferricyanide is used, because of the smaller amounts of reducing material encountered.

The solution is to be prepared in the manner directed in our previous paper.

*Oxalic Acid.*—0.1 N solution.

*Hydrazine Solution.*—Same as previously used (1928).

*Lloyd's Reagent.*—The preparation of fullers' earth known by the above name.

### Procedure for Urine Analysis.

*Preparation of Urine for Analysis.*—Creatinine, uric acid, and other non-glucose materials exert reducing effects which combined usually exceed that of the fermentable sugar in non-diabetic urine. The amount of such substances present is diminished by treatment of the urine with Lloyd's reagent, as described by Folin and Berglund (1922). The substitution of oxalic acid for the sulfuric acid used by them obviates formation of a calcium salt precipitate when the urine filtrate is later mixed with ferricyanide-carbonate solution.

To 10 cc. of urine add 5 cc. of 0.1 N oxalic acid, 5 cc. of water, and 1.5 gm. of Lloyd's reagent. Shake gently for 2 minutes and filter.

A control filtrate is also made at the same time: Add 5 cc. of 0.1 N oxalic acid and 1.5 gm. of Lloyd's reagent to 15 cc. of water, shake for 2 minutes, and filter.

*Determination of Total Reducing Material in Urine Filtrate.*—2 cc. portions of the filtrate are mixed with 2 cc. portions of the ferricyanide reagent in test-tubes, and the analysis is carried out as described for diluted urine on p. 751 of our previous paper (1928), in all respects save the difference in ferricyanide reagent. The $p_0$ value is determined by like analysis of the control filtrate.

### Determination of Non-Fermentable Reducing Material in Urine Filtrate.

A second portion of urine is treated as follows: To 10 cc. of urine are added 7.5 cc. of a 40 per cent yeast suspension (20 gm. of a Fleischmann's compressed yeast cake in 50 cc. of water. The mixture contains, as shown by centrifuging, about 40 per cent by volume of moist yeast). The mixture is allowed to stand for 15 minutes. 5 cc. of 0.1 N oxalic acid and 1.5 gm. of Lloyd's reagent are added. The mixture is shaken for 2 minutes and fil-

tered. Under these conditions, the yeast will remove glucose in amounts up to 0.5 per cent in the original urine.

2 cc. portions of the filtrate are analyzed as in the determination

TABLE I.

*Correction to $p_0$ for Temperature Change.*

| Temperature range. | Increase of vapor tension of water per 1° temperature rise. | Increase of $N_2$ pressure of control per 1° temperature rise. | Total $p_0$ correction per 1 temperature rise.[*] |
|:---:|:---:|:---:|:---:|
| °C. | mm. | mm. | mm. |
| 15–20 | 0.7 | 0.6 | 1.3 |
| 20–25 | 1.2 | 0.6 | 1.8 |
| 25–30 | 1.6 | 0.6 | 2.2 |

[*] Add correction to $p_0$ when temperature is higher at $p_1$ reading; subtract the correction when temperature is lower.

TABLE II.

*Factors by Which $N_2$ Pressure Fall, $p_0 - p_1$ in Millimeters, Is Multiplied to Calculate Reducing Sugar of Urine in Terms of Grams Glucose per 100 Cc.*

| Temperature of gas chamber. | Factor.[*] | Temperature of gas chamber. | Factor.[*] |
|:---:|:---:|:---:|:---:|
| °C. | | °C. | |
| 15 | 0.00143 | 25 | 0.00138 |
| 16 | 2 | 26 | 8 |
| 17 | 2 | 27 | 7 |
| 18 | 1 | 28 | 7 |
| 19 | 1 | 29 | 6 |
| 20 | 0 | 30 | 6 |
| 21 | 0 | 31 | 5 |
| 22 | 0.00139 | 32 | 5 |
| 23 | 9 | 33 | 4 |
| 24 | 8 | 34 | 4 |

[*] These factors hold when, as in the procedure described, undiluted urine is used, and the final 3 cc. of ferricyanide-urine mixture used for the gasometric determination represent 0.75 cc. of urine. If, by reason of high sugar content, the urine is diluted before analysis, the above factors are multiplied as many times as the urine is diluted.

of the total reducing material. The $p_0$ value is determined by similar analysis of filtrate from a control suspension of yeast and Lloyd's reagent, in which water replaces the urine.

The proportions of yeast and fluid are designed to give the same

concentration of non-fermentable reducing material in the fluid that is occasioned, in the preceding total reducing material determination, by diluting the 10 cc. of urine to 20 cc. with water solutions. In the present yeast-fluid mixture, 10 cc. of urine are mixed with 9.5 cc. of water solutions and 3 cc. of yeast cells. The latter, as will be shown, take up about as much non-fermentable reducing material as would the 0.5 cc. of water required to make the fluid volume up to 20 cc.

*Calculation of Fermentable Urine Sugar.*—The results of the two analyses are calculated by means of the factors in Table II, which is constructed from data given in the authors' previous paper (1928). If temperature change occurs between $p_0$ and $p_1$ readings, $p_0$ is corrected according to Table I. The fermentable sugar is calculated as

Fermentable sugar = (total sugar) − (non-fermentable sugar)

TABLE III.

*Impermeability of Yeast to Non-Fermentable Reducing Substances of Blood Filtrate.*

| Filtrate No. | Mg. non-fermentable reducing substances calculated as glucose per 100 cc. blood. | |
| --- | --- | --- |
| | Blood filtrate treated with one portion of yeast. | Blood filtrate treated with two successive portions of yeast. |
| 1 | 19.5 | 20.3 |
| 2 | 19.9 | 19.2 |
| 3 | 20.8 | 22.0 |
| 4 | 25.6 | 28.1 |

EXPERIMENTAL.

*Test of Permeability of Yeast for Non-Fermentable Reducing Substances of Blood Filtrate.*

To ascertain whether measurable amounts of the non-fermentable reducing material in blood filtrate diffuse into yeast cells the following experiment was tried. 1 volume of blood was precipitated with 1 volume of $\frac{2}{3}$ N sulfuric acid, 1 volume of 10 per cent sodium tungstate, and 2 volumes of distilled water, making, in order to obtain a higher concentration of the substances determined, a 5-fold dilution of the blood instead of the usual 10-fold dilution. This filtrate, after treatment with washed, centrifuged

yeast as described above, to remove the fermentable sugar, was divided into two portions. One portion was analyzed to determine the amount of non-fermentable reducing material. The second portion was added to an equal volume of washed packed yeast in a centrifuge tube, was mixed with the yeast, and was allowed to stand for 15 minutes. The yeast was then thrown down by centrifugation and the supernatant solution was analyzed for reducing substances. It is seen from Table III that the amounts of non-fermentable reducing substances in the two filtrates were the same. Consequently there was not sufficient water adherent to the centrifuged yeast to dilute measurably the blood filtrate, nor did the yeast absorb non-fermentable reducing substances from the blood filtrate. Either occurrence would have lowered the concentration of reducing substances in the filtrate.

*Permeability of Yeast for Non-Fermentable Substances of Urine.*

Normal urines were treated with yeast according to the method described above, under conditions assuring complete removal of glucose. The filtrates were then mixed with equal volumes of yeast which had been washed free of reducing substances and centrifuged free of adherent water. The mixtures were centrifuged, and the reducing substances were determined in the supernatant solutions. If the non-glucose urinary reducing substances had been, like those of the blood, not measurably diffusible into the yeast, this second treatment would not have altered the content of reducing substances. If, on the other hand, these substances had distributed themselves uniformly, volume for volume, between yeast and urine filtrate, the second yeast treatment would have lowered by 50 per cent the concentration of reducing material in the urine filtrate. From Table IV it is apparent that neither extreme is realized: the concentration of reducing material in the filtrate was lowered by an average of about 16 per cent, from which there were some wide deviations. According to the average result, non-fermentable reducing materials distributed themselves in about the ratio:

$$\frac{\text{Non-fermentable urinary reducing substances per cc. yeast}}{\text{Non-fermentable urinary reducing substances per cc. urine filtrates}} = \frac{1}{5}$$

Gasometric Fermentable Sugar

*Estimation of Fermentable Sugar in Blood Filtrates from Amount of Reducing Material Removed by Yeast. Comparison of Results Obtained with Different Reagents Used for Determining the Reducing Material.*

Comparison of results obtained by the Benedict copper-colorimetric (1928), Folin ferricyanide-colorimetric (1928), Shaffer-

TABLE IV.

*Permeability of Yeast to Non-Fermentable Substances of Urine Filtrate.*

| | Non-fermentable reducing substance in glucose equivalents. | | | |
|---|---|---|---|---|
| Sample No. | Determined after first yeast treatment. | Determined after treating filtrate from yeast-treated urine with equal volume of moist yeast. | Removed by second yeast treatment. | Proportion of reducing material removed by second yeast treatment. |
| | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *per cent* |
| 1 | 0.112 | 0.101 | 0.011 | 9.8 |
| 2 | 0.096 | 0.078 | 0 018 | 18.7 |
| 3 | 0.117 | 0.096 | 0.021 | 18.0 |
| 4 | 0.053 | 0.042 | 0 011 | 20.7 |
| 5 | 0.032 | 0.032 | | 0.0 |
| 6 | 0.128 | 0 120 | 0.008 | 6.2 |
| 7 | 0.034 | 0.033 | 0.001 | 2.9 |
| 8 | 0 075 | 0.057 | 0.018 | 24.0 |
| 9 | 0.177 | 0 138 | 0.039 | 22.0 |
| 10 | 0.020 | 0.020 | 0.000 | 0.0 |
| 11 | 0.042 | 0 036 | 0.006 | 14.3 |
| 12 | 0.170 | 0 134 | 0.036 | 21.2 |
| 13 | 0.129 | 0.102 | 0 027 | 20.9 |
| 14 | 0.077 | 0.062 | 0.015 | 19.5 |
| 15 | 0.114 | 0.091 | 0.023 | 20.2 |
| 16 | 0.107 | 0 086 | 0.021 | 19.6 |
| 17 | 0.051 | 0.044 | 0.007 | 13.7 |
| 18 | 0.096 | 0.081 | 0.015 | 15.6 |
| 19 | 0.109 | 0.082 | 0.027 | 24.8 |
| 20 | 0.049 | 0.039 | 0.010 | 20.4 |

Hartmann-Somogyi copper-titration (Somogyi, 1926), and the Van Slyke-Hawkins (1928) ferricyanide-gasometric blood sugar methods in analyses of fourteen bloods, normal and pathological, is shown in Fig. 1. In the case of each blood aliquot parts of one portion of Folin-Wu filtrate were analyzed by the respective
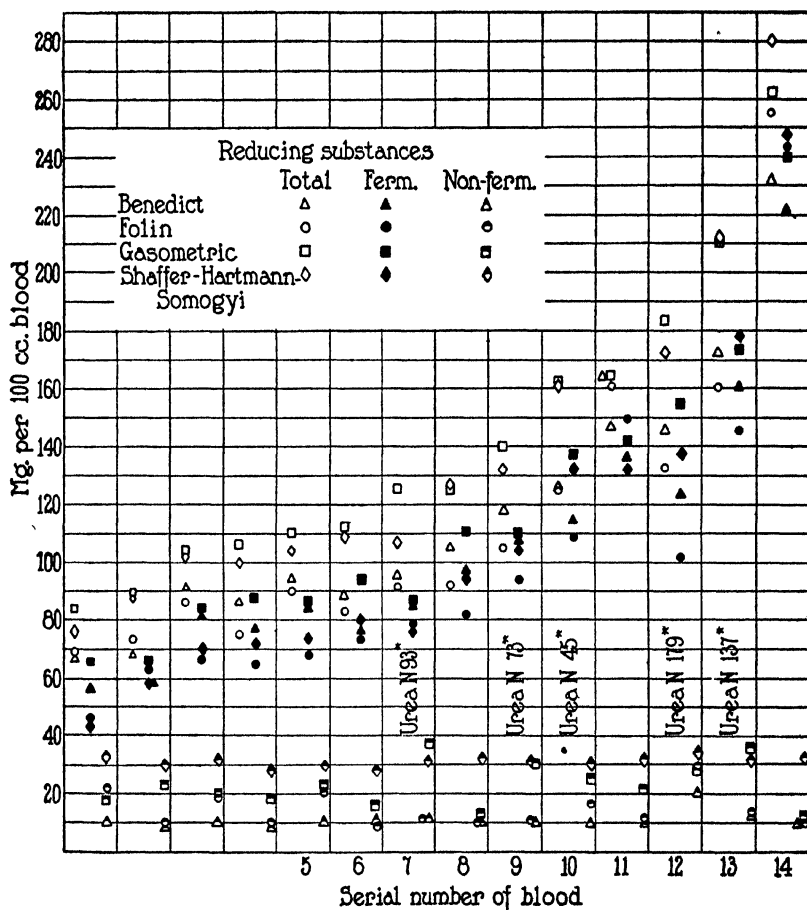
Fig 1 Comparison of total, fermentable, and non-fermentable sugar found in blood by the Benedict copper-colorimetric, Folin ferricyanide-colorimetric, Shaffer-Hartmann-Somogyi copper-titration, and Van Slyke-Hawkins ferricyanide-gasometric methods. Ordinates represent mg. of sugar per 100 cc. of blood. Each symbol represents an average of duplicate determinations.

methods. The fermentable sugar was then removed from another portion of filtrate by treatment with washed yeast as described above, and aliquot parts of the fermented filtrate were analyzed by the same methods.

The values for non-fermentable reducing material varied with the reagent used. Benedict's method gave the lowest and most constant value, averaging about 10 mg. per 100 cc. of blood. The Shaffer-Hartmann-Somogyi method gave about 30 mg. Folin's method gave values varying from 10 to 30 mg. The gasometric method gave values of about 20 mg. per 100 cc. except in three bloods with advanced urea retention. In these cases the non-fermentable reducing materials determinable by the gasometric ferricyanide reagent were increased to over 30 mg., presumably because of retained substances such as creatinine and uric acid, which were shown by Hagedorn and Jensen (1923) to reduce ferricyanide appreciably. The maximum non-fermentable reduction, in a blood with 193 mg. of urea N per 100 cc., was equivalent to 38 mg. per cent of glucose.

The fermentable blood sugar values, if they represented exactly glucose, would be expected to be independent of the reduction method used, as Somogyi and Kramer (1928) have recently found, in fact, with two different copper methods and one ferricyanide method. We have not obtained quite such concordant results. Both the Benedict (1928) and Shaffer-Hartmann-Somogyi (Somogyi, 1926) methods indicated in our hands about 10 mg. per cent less of fermentable sugar than the gasometric ferricyanide method, and the Folin (1928) ferricyanide method averaged still lower.

If one could assume entire absence of technical error, such results would indicate that yeast removes from blood filtrate small amounts of *non-glucose* reducing substances, towards which the reagents of the above respective methods react differently. On the amount of work presented, we hesitate to consider that conclusion as established.

*Determination of Fermentable Sugar in Blood by Spontaneous Glycolysis.*

Comparison of results obtained by Somogyi's (1926) modification of the Shaffer-Hartmann (1920–21) copper reduction method,

with those yielded by the gasometric blood sugar method in analyses of four bloods, normal and pathological, is shown in Table V.

*Fermentable and Non-Fermentable Reducing Substances in Urine of Normal Men, Determined by Gasometric Ferricyanide Method.*

In Table VI are given a number of determinations by the gasometric reduction method described above, of the total and fermentable reducing substances in urine from normal men.

Our values for fermentable reducing sugar in the urines of normal men are mostly between 0.009 and 0.023 per cent, one out of twenty-seven being below and three above this range. Eagle's

TABLE V.

*Determination by Glycolysis of Fermentable and Non-Fermentable Reducing Substances in Blood with the Shaffer-Hartmann-Somogyi and Gasometric Methods.*

Mg. reducing substances per 100 cc. blood.

| Shaffer-Hartmann-Somogyi method. | | | Gasometric method. | | |
|---|---|---|---|---|---|
| Total. | Fermentable | Non-fermentable. | Total. | Fermentable. | Non-fermentable. |
| 122 | 85 | 37 | 116 | 85 | 31 |
| 110 | 86 | 24 | 104 | 82 | 22 |
| 140 | 112 | 28 | 135 | · 114 | 21 |
| 122 | 86 | 36 | 126 | 88 | 38 |

(1926–27) values are all below 0.01 per cent, even when corrected for the effect of yeast volume, discussed above. Eagle determined reducing material with the Benedict copper reagent, while we used a ferricyanide reagent. Whether this difference in reagents is the cause of the apparent difference in results is uncertain. In any case, it appears that the fermentable sugar in normal urine is usually less than 0.025 per cent.

*Fermentable Sugar in Urine Determined by Measurement of $CO_2$ Formed by Yeast.*

Fermentable sugar in the urine was also determined by the measurement of the carbon dioxide formed in urine incubated with

yeast.   A technique was used similar to that employed by one of us (Van Slyke, 1927) for determining urea by measurement of the $CO_2$ yielded by the action of urease.

TABLE VI.

*Content of Fermentable and Non-Fermentable Sugar in Urine of Normal Subjects about 2 Hours after Mixed Breakfast*

| Subject No | Fermentable sugar determined by $CO_2$ formation | Sugar concentration by gasometric reduction method | | |
|---|---|---|---|---|
| | | Total determined (a) | Non-fermentable, determined after yeast treatment (b) | Fermentable, calculated by difference (a) − (b) |
| | *gm per 100 cc* | *gm per 100 cc* | *gm per 100 cc* | *gm per 100 cc* |
| 1 | 0 077 | 0 270 | 0 242 | 0 028 |
| 2 | 0 003 | 0 058 | 0 054 | 0 004 |
| 3 | 0 026 | 0 140 | 0 128 | 0 012 |
| 4 | 0 099 | 0 199 | 0 136 | 0 063 |
| 5 | 0 026 | 0 171 | 0 155 | 0 016 |
| 6 | 0 066 | 0 199 | 0 185 | 0 014 |
| 7 | 0 024 | 0 160 | 0 149 | 0 011 |
| 8 | 0 027 | 0 155 | 0 136 | 0 019 |
| 9 | | 0 185 | 0 178 | 0 007 |
| 10 | | 0 079 | 0 075 | 0 004 |
| 11 | | 0 050 | 0 041 | 0 009 |
| 12 | | 0 133 | 0 120 | 0 013 |
| 13 | | 0 135 | 0 120 | 0 015 |
| 14 | | 0 150 | 0 131 | 0 019 |
| 15 | | 0 140 | 0 127 | 0 013 |
| 16 | | 0 189 | 0 150 | 0 039 |
| 17 | | 0 126 | 0 115 | 0 011 |
| 18 | | 0 108 | 0 098 | 0 010 |
| 19 | | 0 115 | 0 094 | 0 021 |
| 20 | | 0 195 | 0 175 | 0 020 |
| 21 | | 0 116 | 0 104 | 0 012 |
| 22 | | 0 181 | 0 166 | 0 015 |
| 23 | | 0 162 | 0 141 | 0 021 |
| 24 | | 0 064 | 0 045 | 0 019 |
| 25 | | 0 159 | 0 138 | 0 021 |
| 26 | | 0 300 | 0 277 | 0 023 |
| 27 | | 0 080 | 0 071 | 0 009 |

10 cc. of urine were added to 2 cc. of a 30 per cent suspension of washed Fleischmann's yeast in a 20 cc. volumetric flask.   (The proportion of yeast was kept smaller than in the fermentation-

reduction method, in order to minimize the correction due to respiratory $CO_2$ formation in the yeast.) The flask was closed with a 1-hole rubber stopper, the hole of which was filled with a vaselined glass rod. The contents of the flask were mixed, and allowed to stand 1 hour for the yeast to act. The glass rod was withdrawn from the stopper and 8 drops of approximately $CO_2$-free N NaOH were added through the hole by means of a finely drawn out capillary delivery tip. The contents of the flask were made up to the mark and shaken to mix the alkali with the solution and to absorb any $CO_2$ that might have escaped into the gas space beneath the stopper. 5 cc. of the yeast-urine suspension, equivalent to 2.5 cc. of urine, were transferred to the Van Slyke-Neill blood gas apparatus. The suspension was acidified with 2 cc. of 5 N $H_2SO_4$, and the $CO_2$ was determined as described by Van Slyke and Neill (1924). The amount of $CO_2$ in the sample was calculated in mg. by the factors of Van Slyke and Sendroy (1927).

From the total $CO_2$ found in the above analysis, two corrections were determined and subtracted: (a) for the $CO_2$ preformed in the urine, yeast, and alkali used, (b) for the $CO_2$ formed by the yeast from its own substance during the period of fermentation. (a) was determined by $CO_2$ analysis of a mixture of urine + alkali + yeast put together in the order given so that the alkali inhibited action of the yeast on the urine. (b) was determined by mixing yeast with water in place of urine, and determining, by analyses at once and after an hour, the amount of $CO_2$ formed. This amount was relatively small. We assumed that yeast mixed with urine formed from yeast material the same amount of $CO_2$ as yeast mixed with water. If this assumption was erroneous, because of retarding or accelerating effect of urinary substances on the yeast's inner metabolism, it is not probable that the error introduced was important, because the entire value of the (b) correction was small.

With corrections (a) and (b) fermentable sugar was calculated as:

Mg. fermentable sugar per 100 cc. urine = $40 K$ (total mg. $CO_2 - a - b$)

where $K$ represents mg. of glucose yielding 1 mg. of $CO_2$.

The factor 40 is introduced because the samples analyzed each represent 2.5 cc., or $\frac{1}{40}$ of 100 cc., of urine.

The value of $K$ was determined by incubating in the above manner with a portion of the same yeast 10 cc. of a 0.1 per cent glucose solution. The only blank required was the determination of the $CO_2$ in a control yeast suspension in water incubated for the same period. The $CO_2$ in the blank represented preformed $CO_2$ in the yeast + $CO_2$ formed from yeast substance during the time used for fermentation. The $CO_2$ found in the blank was subtracted from that found in the yeast-glucose mixture. The difference represented $CO_2$ formed from 2.5 mg. of glucose. Hence $K$ was calculated as

$$K = \text{mg. glucose yielding 1 mg. } CO_2 = \frac{2.5}{\text{mg. } CO_2 \text{ formed from 2.5 mg. glucose}}$$

TABLE VII.

*Example of Calculation of Fermentable Urine Sugar from $CO_2$ Formed by Yeast.*

|  | $P_{CO_2}$ at 2.0 cc. volume. | Temperature. | $CO_2$ from sample analyzed, equivalent to 2.5 cc. urine. |
|---|---|---|---|
|  | *mm.* | *°C.* | *mg.* |
| Total $CO_2$.......................... | 176.9 | 25.0 | 0.968 |
| Blank ($a$)................. ....... | 37.4 | 25.0 | 0.205 |
| "    ($b$).......................... | 4.5 | 25.0 | 0.025 |

Mg. fermentable sugar per 100 cc. urine = $40\,K$ (total mg. $CO_2 - a - b$)
     = 105.6 (0.968 − 0.205 − 0.025)
     = 77.9
Per cent fermentable sugar in urine  = 0.078
$K$ was 2.64 for the yeast used.

The value of $K$ varied, for Fleischmann's yeast from different cakes, from 2.88 to 2.58. If the fermentation ran entirely in accordance with the equation

$$C_2H_{12}O_6 = 2\,CO_2 + 2\,C_2H_6O$$

the glucose:$CO_2$ ratio would be 180:88 = 2.045. The glucose per mg. of $CO_2$ in our fermentations was found to be from 1.41 to 1.26 greater than this amount. In other words, the $CO_2$ yield was 71 to 79 per cent of the theoretical corresponding to the above equation. An example of the calculation is given in Table VII.

The amount of fermentable sugar in normal urine determined in this manner is seen in Table VI to be from 0.00 to 0.05 grams per 100 cc. greater than that determined by difference in reducing value before and after yeast treatment. In seven of these eight normal urines $CO_2$ was formed in measurable amounts from materials other than glucose. Possibly these materials are amino acids and aliphatic $\alpha$-keto acids, which have been shown (Harden, 1923) to be decomposed by the carboxylase of yeast with evolution of $CO_2$. The amounts of $CO_2$ formed from non-glucose, and

TABLE VIII.

*Content of Fermentable Sugar Determined by $CO_2$ Formation and by Gasometric Reduction Method in Urine of Patients with Definite Glycosuria.*

| Fermentable sugar determined gasometrically by $CO_2$ formation. | Sugar concentration by gasometric ferricyanide method. | | |
| | Total.<br><br>(a) | Non-fermentable, determined after yeast treatment.<br>(b) | Fermentable, calculated by difference.<br>(a) − (b) |
|---|---|---|---|
| *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* |
| 0.263 | 0.391 | 0.117 | 0.274 |
| 0.282 | 0.355 | 0.065 | 0.290 |
| 0.354 | 0.476 | 0.127 | 0.349 |
| 0.640 | 0.794 | 0.089 | 0.705 |
| 0.682 | 0.835 | 0.152 | 0.683 |
| 1.302 | 1.261 | 0.068 | 1.193 |
| 1.318 | 1.223 | 0.118 | 1.105 |
| 1.450 | 1.608 | 0.118 | 1.490 |

probably non-sugar, substances are obviously great enough to invalidate measurement of $CO_2$ formed by commercial yeast as a precise method for determining the slight amounts of glucose in normal urine.

In cases of definite glycosuria (urines with positive qualitative Benedict sugar test) the $CO_2$ method yields, as shown in Table VIII, results approximating those of the reduction method. In such urines the ratio of glucose to interfering substances is so large that the effect of the latter becomes relatively unimportant.

*Effect of Ingestion of Glucose upon Urine Sugar Excretion of Normal Men.*

The subjects of this study were given no food or fluids for a 12 hour period preceding the determinations.  Each subject was given 200 cc. of water at 7.00 a.m. and the urine was collected for

TABLE IX.

*Excretion of Fermentable and Non-Fermentable Reducing Substances after Glucose Ingestion by Normal Men.*

| Subject No. | Time after feeding 1 gm. glucose per kilo body weight. | Urine volume | Concentration of reducing substances in urine. | | | Excretion rate of reducing substances. | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total. | Non-fermentable, determined after yeast treatment | Fermentable, calculated by difference. | Total. | Non-fermentable, determined after yeast treatment. | Fermentable, calculated by difference. |
| | | | (a) | (b) | (a) − (b) | (c) | (d) | (c) − (d) |
| | *hrs.* | *cc. per hr* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *mg. per hr.* | *mg. per hr.* | *mg. per hr.* |
| 1 | −1 to 0 | 64 | 0 079 | 0.075 | 0.004 | 51 | 48 | 3 |
| | 0 " 1 | 26 | 0.103 | 0.101 | 0 002 | 27 | 26 | 1 |
| | 1 " 2 | 94 | 0.090 | 0.084 | 0.006 | 85 | 79 | 6 |
| | 2 " 3 | 218 | 0.025 | 0.025 | 0.000 | 55 | 55 | 0 |
| | 3 " 4 | 198 | 0.027 | 0 025 | 0.002 | 53 | 50 | 3 |
| 2 | −1 " 0 | 28 | 0.185 | 0.178 | 0.007 | 52 | 50 | 2 |
| | 0 " 1 | 27 | 0.203 | 0.187 | 0.016 | 55 | 51 | 4 |
| | 1 " 2 | 32 | 0.168 | 0.153 | 0.015 | 54 | 49 | 5 |
| | 2 " 3 | 25 | 0.154 | 0.144 | 0.010 | 39 | 36 | 3 |
| | 3 " 4 | 26 | 0.171 | 0.158 | 0.013 | 44 | 41 | 3 |
| 3 | −1 " 0 | 31 | 0.123 | 0.114 | 0.009 | 38 | 35 | 3 |
| | 0 " 1 | 31 | 0.110 | 0.093 | 0.017 | 34 | 29 | 5 |
| | 1 " 2 | 39 | 0.101 | 0.088 | 0.013 | 39 | 34 | 5 |
| | 2 " 3 | 24 | 0.126 | 0.114 | 0.012 | 30 | 27 | 3 |
| | 3 " 4 | 16 | 0.169 | 0.154 | 0.015 | 27 | 25 | 2 |

the period from 7.00 to 8.00 a.m.  Glucose was given in 200 cc. of water at 8.00 a.m.  Urine specimens were collected after 1, 2, 3, and 4 hours.  The amount of glucose administered was 1 gm. for each kilo of ideal weight, estimated for the subject's height by Fig. 1 of McIntosh, Möller, and Van Slyke (1928).  The glucose used was Merck's c.p.

The fermentable sugar in the urines of these subjects (Table IX) remained below 0.02 per cent throughout the test period. The data on excretion of urine sugar after the ingestion of glucose confirm the findings of Eagle (1926–27) that fermentable sugar does not usually increase in the urine after ingestion of such quantities of glucose by the normal individual.

### SUMMARY.

Procedures are described for applying the authors' gasometric ferricyanide reduction method to determination of fermentable sugar in blood and urine. Fermentable sugar is measured by the decrease in reducing material caused by brief contact with yeast under conditions producing complete removal of glucose.

The usual normal urine contains about 0.15 ± 0.10 per cent of total reducing substances, of which on the average only about one-tenth is fermentable. Only three urines out of twenty-seven showed more than 0.023 per cent of fermentable sugar. Our normal fermentable urine sugar values are somewhat higher than Eagle's (1926–27), which were below 0.01 per cent, but confirm him in showing that only a relatively slight proportion of the reducing material usually encountered in normal urine can be glucose.

The ingestion of 1 gram of glucose per kilo by three normal men did not in any of them increase significantly the concentration or output rate of fermentable sugar in the urine. This result again confirms Eagle.

Manometric measurement of the $CO_2$ formed by yeast indicated in normal urine fermentable sugar contents averaging 0.03 per cent higher than those estimated from the decrease in reducing power. The extra $CO_2$ is attributable to non-glucose urine constituents, of which amino acids and $\alpha$-keto acids have previously been shown to yield $CO_2$ under influence of yeast carboxylase.

In glycosuric urines the amount of extra $CO_2$ formed by yeast from non-glucose substances is relatively unimportant, and in such urines results by the $CO_2$ method do not deviate significantly from those by the fermentation-reduction method.

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# THE INFLUENCE OF HEMOCYANIN ON THE DISTRIBU-
## TION OF CHLORIDE BETWEEN SEA WATER AND
## THE BLOOD OF LIMULUS POLYPHEMUS.

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Many investigators have observed that the salt concentration of the blood of some marine invertebrates is not equal to that of the particular marine environment from which they have been taken. Duval (1925) has recently reviewed the literature on the relationship between the internal and external environments of aquatic animals and has reported an extensive series of his own observations as well. From his work, which is in general agreement with that of others, he separates the marine invertebrates into two groups. In both groups the blood or body fluid is exactly isotonic with sea water and this isotonicity is maintained when the sea water is concentrated. In one group (echinoderms and worms) the internal environment contains the same concentration of sodium chloride as sea water, and when the sea water is diluted, isotonicity is preserved and the sodium chloride concentration of the internal environment becomes equal to that of the diluted sea water.[1] In the other group (gastropods, cephalopods, and crustaceans) the sodium chloride concentration is about 0.9 that of sea water, and the proportion remains about the same when the sea water is diluted However, while the freezing point of the internal environment decreases as the sea water is diluted, it never becomes as near zero as that of the diluted sea water, even though the animals are given several days in which to reach equilibrium. He notes that the inequality of sodium chloride distribution remains even if he assumes that the blood contains only 95 per cent of water.

[1] Actually he determined chloride only, but he expresses his results as sodium chloride.

The present investigation was undertaken to see whether in a suitable animal the unequal distribution of chloride could be explained on the basis of the Donnan law for membrane equilibria (Donnan, 1924). The blood of the horseshoe crab, *Limulus polyphemus* was chosen because of the ready availability of this animal and the large volume of its blood, and also because other related work being done on *Limulus* blood supplied certain necessary data. Furthermore from the work of Macallum (1910), showing that the composition of *Limulus* blood and sea water was very similar, it seemed probable that the gill membranes of *Limulus* were freely permeable to the elements in sea water.

The ability of the membrane equilibrium to explain the distribution of electrolytes between *Limulus* blood and sea water may be tested by comparing the ratio of any univalent ion in the blood and sea water with the ratio predicted from a consideration of the base-binding power of hemocyanin and the experimentally determined concentration of any univalent ion in either blood or sea water. In this investigation the chloride ratio has been experimentally determined and is compared with the ratio calculated from the sea water chloride and the base bound by hemocyanin.

The accurate calculation of this ratio would necessitate data on many more substances than were actually determined. Chloride of serum and sea water, hemocyanin in serum, and water content of serum and sea water were determined. Van Slyke, Wu, and McLean (1923) summarized the solution laws and facts about the blood electrolytes and derived certain equations for the distribution of electrolytes between cells and plasma, a system comparable to the present one. In order to approximate the theoretical ratio, I have assumed that similar conditions exist in the *Limulus* serum-sea water system, and have derived an equation for the theoretical ratio upon the assumptions which follow.

1. Although Donnan's law predicts the activity ratio of diffusible ions, it is assumed that concentrations may be substituted for activities without introducing an important error, thus:

$$r = \frac{(Na^+)_s}{(Na^+)_{sw}} = \frac{(K^+)_s}{(K^+)_{sw}} = \frac{(H^+)_s}{(H^+)_{sw}} = \frac{(Cl^-)_{sw}}{(Cl^-)_s} = \frac{(B^+)_s}{(B^+)_{sw}} = \frac{(Ac^-)_{sw}}{(Ac^-)_s} \quad (1)$$

where $(Ac^-)$ and $(B^+)$ represent the sum of diffusible univalent

anions and cations respectively and parentheses represent molal concentrations per 1000 gm. of water.

2. Serum and sea water are approximately neutral, thus:

$$(B^+)_{sw} = (A^-)_{sw} \text{ and } (B^+)_s = (A^-)_s \tag{2}$$

where $(A^-)_s$ = the sum of both diffusible and non-diffusible anions of serum.

3. Hemocyanin (Hcy) is the only non-diffusible ion in the system, and the salts which it forms with bases are completely dissociated. The isoelectric point of hemocyanin is on the acid side of neutrality and the acid bound by hemocyanin may be neglected.

4. In the serum the positive charges of the alkali cations are balanced chiefly by diffusible anions which may be represented by $(Cl^-)_s$ and partly by the non-diffusible negatively charged hemocyanin ($Hcy^-$), thus:

$$(A^-)_s = (Cl^-)_s + (Hcy^-)_s = (B^+)_s = (BCl)_s + (BHcy)_s \tag{3}$$

where $(BCl)_s$ represents mols of univalent base equivalent to $(Cl^-)_s$, and $(BHcy)_s$ represents mols of univalent base bound by hemocyanin.

5. $(A^-)_{sw}$ may be represented by $(Cl^-)_{sw}$ without introducing an important error, thus:

$$(A^-)_{sw} = (Cl^-)_{sw} = (B^+)_{sw} \tag{4}$$

6. The effect of bivalent ions may be neglected.

With these assumptions the theoretical ratio may be derived as follows:

Substituting $(Cl^-)_s$ for $(BCl)_s$ in Equation 3

$$(B^+)_s = (Cl^-)_s + (BHcy)_s \tag{5}$$

According to Equation 1

$$\frac{(B^+)_s}{(B^+)_{sw}} = \frac{(Cl^-)_{sw}}{(Cl^-)_s} \tag{6}$$

Substituting in Equation 6 the value of $(B^+)_s$ from Equation 5, and replacing $(B^+)_{sw}$ with $(Cl^-)_{sw}$ according to Equation 4, and rearranging,

$$[(BHcy)_s + (Cl^-)_s] [(Cl^-)_s] = (Cl^-)_{sw}^2 \tag{7}$$

Solving for $(Cl^-)_s$,

$$(Cl^-)_s = \sqrt{(Cl^-)_{sw}^2 + [\tfrac{1}{2}(BHcy)_s]^2} - \tfrac{1}{2}(BHcy)_s \qquad (8)$$

The term $[\tfrac{1}{2}(BHcy)_s]^2$ is negligibly small so we may write

$$(Cl^-)_s = (Cl^-)_{sw} - \tfrac{1}{2}(BHcy)_s \qquad (9)$$

Substituting the above value for $(Cl^-)_s$ in the ratio $r = \dfrac{(Cl^-)_s}{(Cl^-)_{sw}}$ it follows that

$$r = \frac{(Cl^-)_{sw} - \tfrac{1}{2}(BHcy)_s}{(Cl^-)_{s}} \qquad (10)$$

### *Experimental Methods.*

Blood serum was obtained in the manner described by Redfield, Coolidge, and Hurd (1926). The clot, which is composed almost entirely of cells (Loeb, 1909), was removed by centrifuging. Samples of the aquarium sea water in which the animals had been were taken at the same time and preserved in stoppered bottles until analyzed.

Chloride in serum was determined by the method of Van Slyke (1923-24). Owing to the large amount of chloride in *Limulus* blood, it was necessary to make the silver nitrate solution 0.2 N instead of 0.05 N, while the sodium thiocyanate solution was made about 0.1 N. Samples of 5 cc. of serum were used.

Chlorides in sea water were titrated with the F. Mohr method.

The water content of serum and sea water was determined gravimetrically by drying measured samples at 110° to constant weight. 1 liter of sea water was found to contain 986 gm. of water.

Nitrogen was determined by the Kjeldahl method on either 5 or 10 cc. of serum. At first a large number of determinations were made on serum, on the heat-coagulated, washed precipitate of serum, and on the filtrate from the same. The non-protein nitrogen is small, there being only 0.12 gm. of nitrogen non-coagulable by heat in a liter of serum. Determinations were made of the total nitrogen in the serum and the value corrected for non-protein nitrogen by subtracting 0.12. Some of the nitrogen analyses were quite inaccurate and tended to be too low, probably by about 5 per cent, but large differences in (Hcy) make only very slight changes in the calculated ratios.

The concentration of hemocyanin is calculated on the assumption that hemocyanin contains 17.3 per cent of nitrogen (Redfield, Coolidge, and Shotts, 1928), and is expressed as gm. per 1000 gm. of (serum) water.

The base bound by hemocyanin in normal blood has been shown by Redfield, Humphreys, and Ingalls (1929) to vary between $18 \times 10^{-5}$ and $37 \times 10^{-5}$ mols per gm. of hemocyanin. In calculating the theoretical ratio of chloride ions from Equation 10, the concentration of BHcy has been obtained by multiplying the concentration of hemocyanin by $30 \times 10^{-5}$.

## Results.

Table I records the values determined experimentally on ten animals. Concentrations are expressed in gm. of solute per liter of solution. The ratio of serum chloride to sea water chloride is uncorrected for the water content of the blood or sea water. The average ratio is 0.953, a value slightly higher than that obtained by Duval for most invertebrates of the higher group which he studied.

In Table II the concentrations of chloride are expressed in mols per 1000 gm. of water in serum or sea water and the concentration of hemocyanin in gm. per 1000 gm. of (serum) water. The reasons for expressing the values on a salt:water basis, rather than salt:volume basis are discussed by Van Slyke, Wu, and McLean (1923). The average value of $r$ based on the ratio of the observed concentrations of chloride corrected for water is 0.981. In the last column of Table II the values of $r$ calculated from Equation 10 are given; of these the average value is 0.991. The difference of about 1 per cent between the observed and calculated values is probably significant, but the agreement is close enough to suggest that the Donnan equilibrium due to the presence of hemocyanin will account for a considerable part of the inequality in the chloride distribution.

Since both the blood and sea water are relatively concentrated solutions, the original assumption that concentration values may be substituted in the Donnan equilibrium is not valid, and was made only because the activity coefficients of $Cl^-$ in sea water and blood are not known. In the absence of more precise data in the actual experiments, however, it seems undesirable to attempt to

approximate the activity coefficients. A rough calculation of the theoretical ratio, activity coefficients which seemed reasonable

TABLE I.

*Limulus Serum and Sea Water Protein Nitrogen, Chloride, and Water in Gm. per Liter.*

| Animal. | $H_2O$ in serum. | Protein N in serum. | Chloride in serum. | Chloride in sea water. | $\dfrac{\text{Cl in serum}}{\text{Cl in sea water}}$. |
|---------|------------------|---------------------|--------------------|------------------------|------------------------|
|         | *gm. per l.*     | *gm. per l.*        | *gm. per l.*       | *gm. per l.*           |                        |
| a       | 957              | 5.9                 | 17.44              | 18.12                  | 0 962                  |
| b       | 942              | 8.6                 | 16.70              | 18.12                  | 0 921                  |
| c       | 969              | 3.7                 | 17.61              | 18.12                  | 0 972                  |
| d       | 965              | 4 2                 | 17.05              | 18.12                  | 0.941                  |
| e       | 957              | 6 5                 | 17.14              | 18 13                  | 0 945                  |
| f       | 946              | 8 1                 | 17.14              | 18.15                  | 0.944                  |
| h       | 962              | 4 6                 | 17.40              | 18.15                  | 0.958                  |
| i       | 941              | 9 7                 | 17.05              | 18.15                  | 0.939                  |
| j       | 972              | 2 9                 | 17 55              | 18.15                  | 0.967                  |
| k       | 978              | 1 5                 | 17.82              | 18.15                  | 0.981                  |
| Average ........................................... ........ | | | | | 0 953 |

TABLE II.

*Limulus Serum and Sea Water Concentrations of Hemocyanin and Chloride per 1000 Gm. of Water, r Observed and Calculated.*

| Animal. | $(Hcy)_s$ | $(Cl)_s$ | $(Cl)_{sw}$ | r observed. | r calculated. |
|---------|-----------|----------|-------------|-------------|---------------|
|         | *gm. per 1000 gm. $H_2O$* | *mols per 1000 gm. $H_2O$* | *mols per 1000 gm. $H_2O$* | | |
| a       | 36        | 0.513    | 0.518       | 0.991       | 0.991         |
| b       | 53        | 0.499    | 0.518       | 0.964       | 0.985         |
| c       | 22        | 0.512    | 0.518       | 0.989       | 0.995         |
| d       | 25        | 0.498    | 0.518       | 0.961       | 0.993         |
| e       | 39        | 0.504    | 0.518       | 0.972       | 0.989         |
| f       | 49        | 0.510    | 0.519       | 0.983       | 0.987         |
| h       | 28        | 0.509    | 0.519       | 0.981       | 0.993         |
| i       | 60        | 0.510    | 0.519       | 0.983       | 0.984         |
| j       | 17        | 0.508    | 0.519       | 0.980       | 0.995         |
| k       | 8.9       | 0.513    | 0.519       | 0.988       | 0.999         |
| Average................................... | | | | 0.981 | 0.991 |

being used, lowered the theoretical ratio by only 0.005 or 0.006, and this is within the error of the experiments.

## SUMMARY.

1. The concentration of chloride in the serum of *Limulus polyphemus* is 0.953 times the concentration of chlorides in sea water, the concentration being estimated on the basis of the total volume of solution.

2. When the concentration is estimated on the basis of the water in serum and sea water the ratio is 0.981.

3. The Donnan equilibrium due to the hemocyanin of the serum will account theoretically for a considerable part of the difference in chloride concentration between the serum and sea water.

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# BUFFER INTENSITIES OF MILK AND MILK CONSTITUENTS.

## I. THE BUFFER ACTION OF CASEIN IN MILK.*

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### INTRODUCTION.

Titration data on milk have been published by Van Slyke and Baker (1), Buchanan and Peterson (2), Moser (3), and others. Titration curves of milk have been given by Clark (4) and by Moser (3). Buchanan and Peterson (2) have calculated the buffer efficiency of milk for a number of zones of the pH range. Clark (4, 5) has made several similar calculations and has outlined the difficulties to be avoided or surmounted in integrating the buffer activities of the components of milk. It has not been feasible to use any of these data in this work because of the possible differences in composition in the milks employed and because of the non-uniformity of range and frequency in the data reported. Wherever comparisons were possible, the agreement between previous data and those of this paper was satisfactory.

The mathematical theory of buffer action in heterogeneous systems has been developed by Kugelmass (6) and by Klinke (7). While these methods of calculation cannot be applied to hydrogen ion buffering in systems involving casein until the essential constants are known with a fair degree of accuracy, it appears from the general aspect of Klinke's calculations that the effect of phase buffering should be most intense at the hydrogen ion concentra-

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tions at which pH = pK, though the actual values would be different from those for homogeneous buffering.

Data obtained by Buchanan and Peterson (2) show that milk is a more efficient buffer in the range pH 5.0 to 6.0 than it is at higher pH values. The use of phenolphthalein as indicator in the titration of milk is a recognition of the fact that the buffering efficiency of milk is comparatively low in the region pH 7.5 to 8.5.

If the titration curves—cc. of acid or alkali plotted against pH values—of any but the simplest substances are compared, the observable differences are not very striking. The general directions taken by curves for complex mixtures are necessarily much the same, the noticeable differences being in apparently very slight variations in slope. If, however, the slopes are evaluated and plotted against pH values, the resulting curves show striking variations. These slopes are values of what Van Slyke (8) calls the buffer index and may be considered measures of buffer intensity at the various hydrogen ion concentrations indicated by the pH values. Mathematically expressed, the buffer index is the first derivative of the function expressed by the corresponding titration curve; less exactly, it is the change in normality of acid or base necessary to produce a change of hydrogen ion concentration of one pH unit in a zone centered on a given pH value. The area beneath the buffer intensity curve between any two pH values is a measure of the quantity factor, or buffer capacity.

The Van Slyke buffer index, $\dfrac{dB}{dpH}$, may be calculated as the ratio of the change of normality of acid or base to the corresponding change in pH value, the increments being chosen as small as practicable. On purely theoretical grounds it is perhaps more logical, as shown by Leuthardt (9), to express the buffer index as $\dfrac{dB}{d[H^+]} - \left(\dfrac{2[H^+]}{K} + 1\right)$, in which the concentration of the hydrogen ion, rather than its negative logarithm, is used and terms are included to allow for the strength of the acid or base and for the buffering due to water alone. When strong acid or base is used, this expression may be simplified to $\dfrac{dB}{d[H^+]} - 1$. Leuthardt has shown that in the range pH 3.0 to 11.0 the buffering effect of water may be neglected in the Van Slyke formula without sensible

error. Consequently it is immaterial for purposes of comparison which of these formulas is used when the titration is carried out with strong acid and base in this range. However, when values of buffer index are to be plotted against values of hydrogen ion concentration, it becomes necessary for practical reasons to use a logarithmic relationship and it is much simpler to use the Van Slyke index which already embodies such a relationship.

<center>EXPERIMENTAL.</center>

In all continuous titrations on a single sample of milk or of its products, a doubt arises as to whether equilibrium is attained at the time of each successive reading. Obviously, even when preservatives are used, it is not advisable to extend the time of a titration beyond a few days. In order to avoid the difficulty, each pH value determined in this work was obtained from a separate sample, at least 2 hours being allowed for attainment of equilibrium in each case. Furthermore, check determinations were made on a number of samples from each set after successive 24 hour periods to insure that equilibrium had been reached. Only in experiments dealing with calcium phosphate in the absence of casein was a time of 2 hours found to be insufficient. Those experiments are to be discussed in a later paper.

Samples of 25 or 50 cc. were used. The necessary volume of 0.2 N hydrochloric acid or sodium hydroxide solution was added from a pipette in a fine stream while the sample was stirred with an electrically driven agitator. Results of Buchanan and Peterson (2) indicate that dilution of milk up to 30 per cent of its original volume does not appreciably affect its buffer action. This degree of dilution was not exceeded except in the case of milk itself below pH 4.5, a region not ordinarily of much importance in milk equilibrium studies. Furthermore, the effect of dilution was partially compensated for by the method of calculation chosen.

The determinations of hydrogen ion concentration were made in duplicate by the quinhydrone method with gold electrodes of the capillary type described by Cullen and Biilmann (10) and Watson (11). Potential differences against a saturated calomel half-cell were measured with a Leeds and Northrup type K potentiometer.

For purposes of calculation, values were chosen such that the increment of pH change was approximately 0.5 pH unit. It is of course desirable to calculate from values of dpH as small as possible in keeping with the accuracy of the measurements. It was found that the use of increments smaller than 0.35 pH units led to irregularities in the curves,—irregularities which could not be duplicated and which were undoubtedly due to the fact that the safe proportional error was being exceeded. Substitution was made in the formula

$$\frac{dB}{dpH} = \frac{\left(\begin{array}{c}\text{cc. acid or base added} \\ \text{to cause pH change}\end{array}\right)\left(\begin{array}{c}\text{normality factor} \\ \text{of acid or base}\end{array}\right)}{\left(\begin{array}{c}\text{average volume of sample} \\ \text{over range involved}\end{array}\right)\left(\begin{array}{c}\text{pH change} \\ \text{produced}\end{array}\right)}$$

and the resulting value attributed to the pH value at the midpoint of the range involved. In the accompanying figures actual points from which the curves were constructed are not shown, since their loci were determined to a slight degree by choice of range for calculation as well as by the actual measurements. Only the major features of the curves are considered in the discussion of results.

### DISCUSSION.

Results on raw skim herd milk were unexpectedly uniform, the maximum buffer intensity occurring in a zone at about pH 5.5. The data for a representative set of values are plotted as Curve A in Figs. 1, 2, and 3. The effect of "forewarming" milks to various temperatures was slight and not proportional to the degree of heat employed, but was in general to increase the intensity of buffer action between pH 5.0 and 5.5; that is, at hydrogen ion concentrations favorable to coagulation. It is planned to investigate this effect in more detail. Other members of this laboratory staff are determining the extent and significance of variation in buffer intensities of the milk of individual cows and of differences attributable to breed.

Since in the grain curd casein process a comparatively pure casein is precipitated, it was believed that by comparing the buffer intensity curves of skim milk and grain curd casein whey information as to the actual buffering by casein in milk could be obtained. In Fig. 1 it may be noted that the whey curve becomes identical

with the skim milk curve at pH 4 and at about pH 7. The area between the two curves apparently is a measure of the buffer capacity of casein in this pH range. The rise of the whey curve above the milk curve at pH 6.0 cannot be attributed simply to the effect of casein removal, since this rise is not noticed in the case of rennet whey, but is probably due to a change in type of buffering of some inorganic constituent in some way influenced by the action of acid, since it is also apparent in lactic acid whey. The difference between Curves A and B is shown in Fig. 1 by Curve C
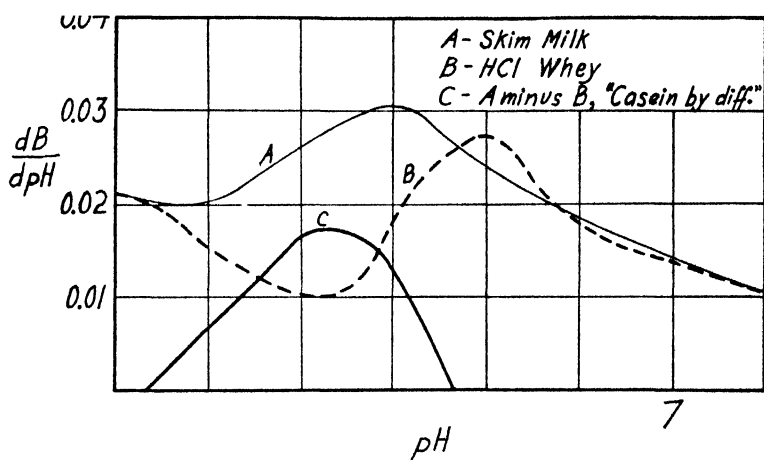


Fig 1. Buffer intensity curves of skim milk, grain curd casein whey, and casein by difference.

plotted from the base line. Presumably this curve represents the buffer intensity of casein as it exists in milk. Its general shape and its position are those to be expected from a pure substance having one or more pK values of 5.2 and no other pK values between 4.0 and 7.5. It is unsafe to base any conclusion on the value of the maximum of this curve without a knowledge of all the pK values and of the extent to which the rôle of casein in milk is of the heterogeneous type.

Whey produced by fermentation of skim milk with a pure culture of *Streptococcus lactis* and subsequent removal of the curd was used in obtaining the results shown by Curve B of Fig. 2.
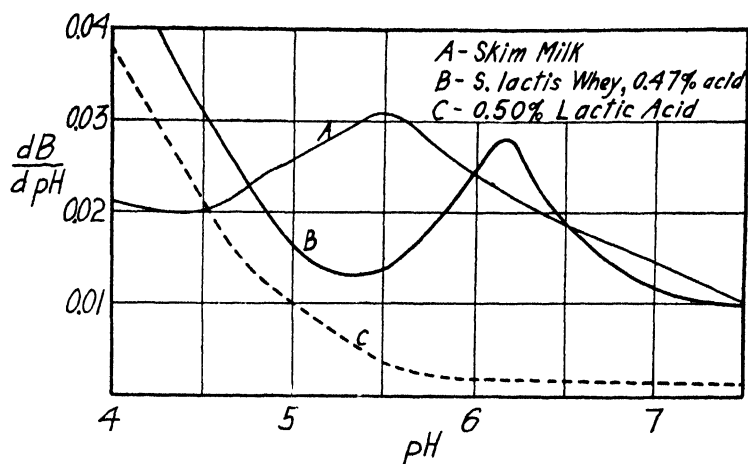
Fig. 2. Buffer intensity curves of skim milk, lactic acid whey, and 0.50 per cent lactic acid solution.
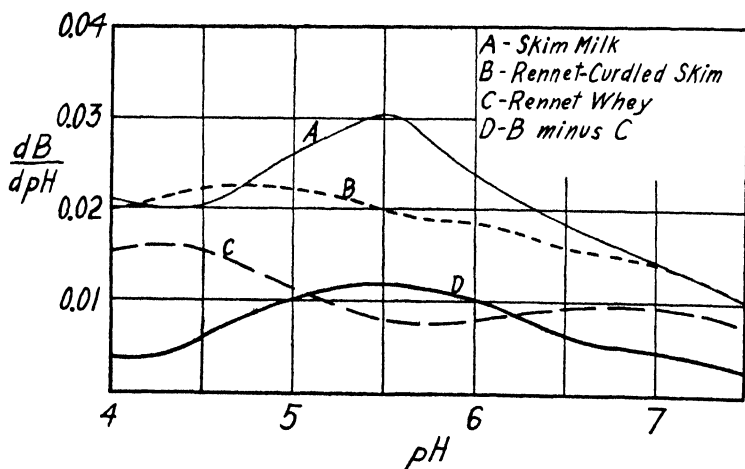


Fig. 3. Buffer intensity curves of skim milk, rennet-curdled skim milk, rennet whey, and paracasein by difference.

Here again is evident the node at about pH 6.0. The steep rise of the curve at lower pH values is due to buffering by the lactate radical, as may be seen by a comparison with Curve C obtained on a water solution of approximately the same percentage lactic acid as the whey. The maximum effect of the lactate should be at pH 3.8. Except for the buffering influence of the lactic acid, Curve B of Fig. 2 is in good agreement with Curve B of Fig. 1.

The effect of rennet curdling is given in Fig. 3. The differences between Curves A and B apparently represent differences between casein and paracasein. The differences between Curves B and C
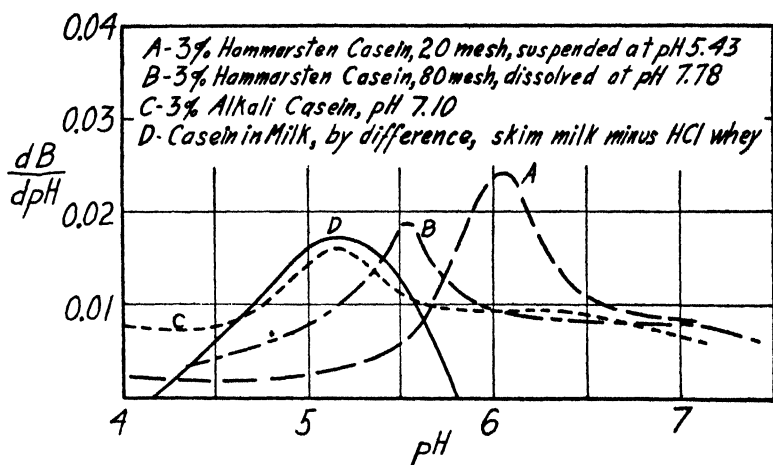


FIG. 4. Buffer intensity curves of caseins.

are due to the absence of the curd in the second case, and these differences plotted from the base line give Curve D which is assumed to be the buffer intensity curve of paracasein in approximately 3 per cent concentration. This curve is quite different from the curve for casein, a number of dissociation constants being indicated for paracasein in the range in which casein exhibits but one. These constants are so close to one another in value that the nodal portions of the curve attributable to each are confused by mutual overlapping. The fact that titration data of Palmer and Richardson (12) indicate for paracasein only one dissociation constant in the range considered here implies either that crude

paracasein is a mixture or that the purification process causes rather profound changes in the paracasein molecule.

The casein curve of Fig. 1 is reproduced in Fig. 4 as Curve D for comparison with curves obtained by using purified casein. Curve A was obtained on a 20 mesh sample of Hammarsten casein added to distilled water to give a concentration of 3 per cent. Much of the material was so coarse that it settled immediately after agitation was stopped. Some of the same lot of casein was ground to pass an 80 mesh screen, and several samples were

TABLE I.

*Relative Buffer Capacities of Milk and Certain Derivatives*

|  | 0 2 N acid required to change pH of 100 cc from 7 0 to 4 0 |
|---|---|
|  | cc |
| Skim milk | 40 |
| Rennet-coagulated skim milk | 32 |
| Rennet whey | 17 |
| "      curd (by difference) | 15 |
| Grain curd casein whey | 28 |
| "      "    (by difference) | 12 |
| *Streptococcus lactis* whey | 34 |
| 0 50 per cent lactic acid | 14 |
| 3 per cent alkali casein          . | 16 |
| 3  "    "   Hammarsten casein, suspended, pH 5 43 | 13 |
| 3  "    "          "          "    dissolved,   "   7 78 | 14 |

suspended in water. With this finer material a number of points on Curve A were checked, the coarser particles evidently having reacted to the same degree as the finer. More of the 80 mesh Hammarsten casein was suspended in water and, with rapid agitation, sodium hydroxide solution was added slowly until individual particles were no longer visible. At no time was the mixture sufficiently alkaline to color phenolphthalein except at the point of addition. After the solution was diluted to give a concentration of 3 per cent casein, its reaction was at pH 7.78. Results calculated from titration of this solution are given as

Curve B. A casein produced commercially for bacteriological use was added to distilled water to give a 3 per cent concentration. A somewhat viscous fluid with a reaction at pH 7.10 was obtained. Results from this casein are given as Curve C. It may be noted that the areas beneath the curves—that is, the buffer capacities—are approximately the same for all caseins used within the range of the plot. The striking feature, however, is the difference in pH values at which maximum buffering occurs. This difference indicates a difference in dissociation constants for any two samples. This evidence, as well as the fact that it has frequently been noted that caseins differ in certain other properties, points to the conclusion that casein is chemically changed under the conditions necessary for its isolation.

Buffer intensities calculated from titration data of Palmer and Richardson (12) on casein and paracasein purified by the method of Van Slyke and Bosworth (13) agree reasonably with the data of Curve A of Fig. 4. Evidently their purified paracasein was a substance with a dissociation constant very nearly the same as that of their casein, and with no other dissociation constants of similar magnitude.

The relative buffer capacities derived from the data used in constructing the curves have been roughly calculated and are given in Table I. They indicate more simply than the graphs, though in much less detail, the contribution of casein to the buffer action of milk.

### CONCLUSIONS.

From titration data on milk a characteristic curve may be obtained by plotting buffer intensity against hydrogen ion concentration expressed in pH units.

Maximum buffering in normal milk occurs at approximately pH 5.50.

The buffer intensity curve of casein determined by difference indicates that the buffer action of casein is exerted principally between pH 4.50 and pH 5.70 with a maximum at approximately pH 5.20. Casein is evidently one of the chief factors in the buffer action of milk in this range.

The buffer intensity curves of purified caseins indicate that

differences in methods of isolation and purification produce caseins differing from one another and from casein as it exists in milk.

The addition of rennet to milk causes the pronounced node in the buffer intensity curve of milk to disappear. Apparently rennet converts casein into a substance with several dissociation constants within the range of pK values from 4.0 to 7.5. This conclusion is supported by the buffer intensity of paracasein determined by difference.

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# TYROSINE AND TRYPTOPHANE DETERMINATIONS IN ONE-TENTH GRAM OF PROTEIN.

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## I. Preliminary Discussion.

In the colorimetric methods for the determination of tyrosine and tryptophane, described by Folin and Ciocalteu (1) in 1927, 1 or 2 gm. of protein material are taken for the preliminary hydrolysis. It has seemed desirable to try to adapt these methods to the use of smaller amounts of material (0.1 gm.) in order to broaden the applicability of the methods. While this work was in progress, there appeared a lengthy paper, by Hanke (2), on colorimetric tyrosine determinations, and it seems suitable first to consider that paper before taking up the constructive work.

Hanke's method for tyrosine was published in 1922 and, during the intervening 7 years, Hanke has repeatedly used that method as a basis for criticism of other methods, particularly those published from this laboratory. He now finds that one essential step in his method, namely the removal of histidine with silver oxide, also removes tyrosine and, on the basis of that error alone, the tyrosine content of casein moves from 4.5 per cent (his earlier figures) to about 5.5 per cent. As the silver oxide treatment was a necessary prerequisite step in his method, he now definitely abandons that method.[1] It is to be noted further that there is now no continuity between his tyrosine work of the past 7 years and the main part of the work reported in his present paper. What he now presents is just a modification of the Folin-Ciocalteu method, and on that

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[1] As late as 1927, Hanke insisted that the tyrosine in the silver oxide precipitates is not tyrosine, but an "X factor," the presence of which proved the superiority of his method. (Hanke, M. T., *J. Biol. Chem.*, **74**, p. x (1927).)

basis he is taking a fresh start in the field of tyrosine determinations.

There is one slight link of continuity between Hanke's past and his present work on tyrosine determination. He still maintains that the tyrosine must be precipitated from the hydrolysates and he uses for that precipitation the same treatment with mercuric acetate and sodium chloride as was used in his own method. It is well that some one should continue to take that

TABLE I.

*Precipitation of Tyrosine from Pure Solutions by Means of Mercuric Acetate and Sodium Chloride.*

| Tyrosine taken | Tyrosine recovered | Difference. | Loss |
|---|---|---|---|
| mg | mg. | mg. | per cent |
| 2 | 1.316 | 0.684 | 34.2 |
| 3 | 1.67 | 1.33 | 44.3 |
| 3 | 2.01 | 0.99 | 33.0 |
| 4 | 3 49 | 0.51 | 12.75 |
| 4 | 2 79 | 1.21 | 30.25 |
| 5 | 4.21 | 0.79 | 15.84 |
| 5 | 3.84 | 1 16 | 23.20 |
| 5 | 3.51 | 1.49 | 29.8 |
| 6 | 4.47 | 1.53 | 25.5 |
| 8 | 7.43 | 0.57 | 7.13 |
| 10 | 8.62 | 1.38 | 13.8 |
| 10 | 8.62 | 1.38 | 13.8 |
| 10 | 9.30 | 0.70 | 7.0 |
| 20 | 19.04 | 0.96 | 4.8 |
| 50 | 47.6 | 2.40 | 4.8 |
| 50 | 48.96 | 1.04 | 2.1 |

view, because it may ultimately lead to some quantitative method for the isolation of tyrosine. But after his unhappy experience with one of his precipitants (silver oxide) one would think that Hanke would not immediately set out on a new venture with the other (mercuric acetate and sodium chloride) without first positively proving that it does quantitatively precipitate tyrosine both from pure solutions and from hydrolysates. Hanke's statement on the subject is not altogether satisfactory and his analytical data are too meager, since they are supposed to prove the most important point in his paper. We have deemed it worth while, therefore,

to make some experiments of our own on the precipitation and re-covery of tyrosine from pure tyrosine solutions, by Hanke's method. Our results are recorded in Table I.

The figures in Table I speak for themselves. But we might call particular attention to the lack of uniformity in the absolute losses of tyrosine. Practically every experiment gives a different loss even when the same amount of tyrosine is taken. It may be pertinent to recall in this connection that Hanke now believes that the tyrosine content of casein is a variable, so that, in its tyrosine content, the casein from one cow is different from the casein obtained from another, and, in addition, that old casein samples contain less tyrosine than when first prepared. One would neces-sarily expect to encounter both larger and more variable losses from protein hydrolysates than from pure tyrosine solutions or from artificial mixtures of pure amino acids, by his precipitation process.

Hanke checked up the losses of tyrosine by determining the tyrosine content of the filtrates from the tyrosine mercury precipi-tates, so that the losses when working with pure solutions could readily be interpreted as representing merely the solubility of the tyrosine precipitate. Our experience does not confirm this obser-vation. Our filtrates yielded only insignificant traces of tyrosine, so that in our work the lost tyrosine had just disappeared. It is to be noted that Hanke when working with casein hydrolysates obtained exactly the same sort of tyrosine disappearance which we have obtained from pure tyrosine solutions. His explanation of the phenomenon seems rather romantic (3).

There is one observation in Hanke's paper for which we must not fail to give him credit; namely, the fact that cystine interferes with the direct determination of tyrosine by the reaction of Folin and Ciocalteu. This seemed of interest to us because our plan of work included a critical study of cystine determinations in acid protein hydrolysates by the method of Folin and Looney. Cystine is destroyed when protein is hydrolyzed with sodium hydroxide just as tryptophane is destroyed by acid hydrolysis. But just as the decomposition products of tryptophane might interfere with the subsequent determination of tyrosine with the phenol reagent by giving some color with it, so the residue from the cystine may interfere with the tyrosine determinations in the new method.

because the residue, like undecomposed cystine, gives a precipitate and turbidities with the mercury reagent.

In the Folin-Ciocalteu paper, due attention was called to the fact that there is danger of getting turbidities when working with protein hydrolysates. It was not recognized that these turbidities were due to the original presence of cystine. The method as described provides, however, not only for the removal of the tryptophane, but also for the removal of most of the decomposed cystine, and if the method is carried out exactly as described, with due attention to the acidities of the reagents, turbidities will rarely be encountered. The precipitate which cystine and the cystine derivative give with mercuric sulfate is the more soluble the greater the acidity. In the Folin-Ciocalteu method, the cystine derivative and the tryptophane are precipitated from a total solution of 12 cc., having an acidity of a little more than 3 N, and the final reading of color is made on 100 cc. of solution whose acidity is that of a normal acid. These conditions were empirically found to yield clear deep red solutions with the protein hydrolysates. But it is possible that the selected conditions provide a little too small a margin of error though this margin was intentionally made small, because the greater the acidity of the final solution the more does the color change from red to orange. The directions also call for an immediate color comparison. It is not really essential that the colors obtained should contain the least possible shade of yellow, for the somewhat more orange-colored solutions obtained at an acidity of about 1.2 N acid also give a perfect proportionality for widely different quantities of tyrosine. By adding 9 cc. of 7 N acid to the standard and the unknown where the original directions call for 6 cc. (p. 139 of the Folin-Ciocalteu paper) the suggested acidity is obtained and the tendency to get turbidities is practically excluded. By the additional change of the heating time from 15 minutes to 5 minutes, all turbidity due to cystine is completely excluded.

In connection with the present research we have, of course, taken cystine into account, as was not done by Folin and Ciocalteu, and have shown that neither the cystine as found in acid hydrolysates, nor added cystine, nor alkali-decomposition products of cystine interfere with our tyrosine and tryptophane determinations.

In other words, the Folin-Ciocalteu process for the determination of tyrosine can be applied to protein hydrolysates obtained by the help of sulfuric acid. The mercuric sulfate precipitation then removes the cystine so completely that it does not interfere with the tyrosine determination. In this case, one simply precipitates the cystine in exactly the same manner as the tryptophane is precipitated from the alkaline hydrolysates. And it may be' remarked in passing that the tyrosine values will be the same whether one uses an acid or an alkali hydrolysate. In the present research, we encountered some difficulty due to cystine when working with serum albumin. This protein contains fully 6 per cent of cystine, a value almost approaching the cystine contents of the keratins, and with the alkali hydrolysates from this protein we did encounter some troublesome turbidities in the tyrosine determinations until we had discovered the twofold remedy suggested above.

## II. Development and Description of Micro Method.

As already indicated, we are here trying to solve the problem of determining both tyrosine and tryptophane in 0.1 gm. of protein. In pursuit of this object, we have replaced Kjeldahl flasks with test-tubes and have substituted the water bath for direct boiling in connection with the hydrolysis. A temperature of about 100° is, of course, much less effective than is boiling with a 20 per cent sodium hydroxide solution, and we were rather skeptical as to whether the projected process would work. And as a matter of fact it does not work with casein. It is remarkable that casein should be so much more resistant to alkaline hydrolyses than other proteins, but the fact that it cannot be used in this process is perhaps of minor importance, since this protein is always available in large quantities.

Our micro method probably could be adapted to casein by substituting gentle boiling in a metal bath or an oil bath for the lower temperature of the steam bath, if one wanted, for example, to make a comparative study of the caseins obtained from different animals, but as we are not now contemplating such a study here, we have not included casein in this work.

Since our micro methods are in principle identical with the original methods of Folin and Ciocalteu we shall confine ourselves to concise descriptions of the methods as actually used.

*Hydrolysis.*—Transfer to the bottom of a clean, dry Pyrex test-tube (150 mm. × 16 mm.) about 100 mg. of dried protein material. Add 2 cc. of 20 per cent sodium hydroxide solution. Shake gently with a little heating until the protein has dissolved. Close the mouth of the test-tube with a cork wrapped in tin-foil and carrying as condenser a glass tube about 50 cm. long. Heat in a steam bath or boiling water bath for 12 to 18 hours. The time required for complete hydrolysis differs for different proteins. Albumins are hydrolyzed in from 12 to 14 hours, but globulins require from 16 to 18 hours. At the end of the hydrolysis, add immediately to the hot solution 3 cc. of 7 N sulfuric acid. It is essential that the silica should here be obtained as a precipitate and not as a colloidal solution. After cooling, transfer the hydrolysate to an accurately graduated 25 cc. test-tube or volumetric flask by repeated washings with small quantities of water. Dilute to volume with water, add 0.2 gm. to 0.5 gm. of kaolin, shake well, and filter. A small filter (9 cm.) should be used, and the funnel must be covered with a watch-glass to prevent evaporation, because the filtration is slow. 20 cc. of the filtrate are taken for the determinations.

*Determination.*—Transfer 20 cc. of the filtrate to a conical centrifuge tube, capacity 50 cc. Add 4 cc. of a solution containing 15 per cent of mercuric sulfate in 6 N sulfuric acid. The mercury solution must be added, drop by drop, from a distance of not less than 3 cm. Perfect mixing is thus secured without any stirring. Set aside for 2 to 3 hours. 2 hours are required for the complete precipitation of the tryptophane. Centrifuge at a fairly high speed for 5 to 10 minutes. We have now the tryptophane with a little tyrosine (and nearly all of the cystine derivative) in the sediment and the tyrosine in the supernatant solution.

Decant into a 100 cc. volumetric flask and wash the edge of the centrifuge tube with 1 cc. of 0.1 N sulfuric acid.

Next transfer 10 cc. of a 1.5 per cent mercuric sulfate solution in 2 N sulfuric acid and stir with a glass rod; rinse the rod with 2 cc. of the same solution, and let the mixture act for 10 minutes. Centrifuge as before, and add the wash liquid to the main solution in the volumetric flask, again rinsing off the lip of the centrifuge tube with 1 cc. of 0.1 N sulfuric acid.

In order to remove the surplus mercury and the slight traces of tyrosine which may still be in the centrifuge tube, we wash the precipitate once more, this time with 0.1 N sulfuric acid. Add 10 cc. of the acid, stir, and wash the stirring rod with 2 cc. of the 0.1 N acid. Centrifuge as before and transfer the supernatant liquid to the volumetric flask.

For the determination of the tyrosine in the volumetric flask, it is first necessary to transfer to another 100 cc. volumetric flask 4 mg. of tyrosine plus the same amounts of water, acid, and mercuric sulfate as are contained in the unknown solution; that is to say (1) 4 cc. of a solution of tyrosine in 2 N sulfuric acid and containing 1 mg. of tyrosine per cc., (2) 16 cc. of water, (3) 4 cc. of the 15 per cent mercuric sulfate solution, (4) 12 cc. of the 1.5 per cent mercuric sulfate solution, (5) 14 cc. of 0.1 N sulfuric acid. Finally add 6 cc. of 7 N sulfuric acid to the contents of each volumetric flask. The acid in each flask should be equivalent to just about 100 cc. of N $H_2SO_4$.

It may be remarked here that we are adding only 6 cc. of 7 N $H_2SO_4$, although we recommend the addition of 9 cc. of 7 N acid at the same stage in the original Folin-Ciocalteu process. The difference is due to the fact that the tryptophane is not precipitated at the same degree of acidity in the two procedures.

Heat the contents of the two flasks simultaneously in boiling water for 5 minutes. Folin and Ciocalteu prescribe a heating period of 15 minutes but 5 minutes are ample. Cool the flasks. Add, with shaking, 1 cc. of 2 per cent sodium nitrite solution to each flask and after 2 minutes waiting dilute to volume, mix, and make the color comparison without undue delay.

4 × 20, or 80, divided by the colorimetric reading obtained (when the standard is set at 20 mm.) gives in mg. the amount of tyrosine present in the 20 cc. of hydrolysate which were taken for analysis.

Some supplementary remarks should be made.

1. The acidity of the unknown and the standard, after dilution to 100 cc., should be very nearly the same. The maximum permissible difference is 10 per cent; that is, if the acidity of the standard is normal, that of the unknown must be between 0.9 N and 1.1 N.

2. Perceptible fading begins within 30 minutes.

3. In the Folin-Ciocalteu reaction for tyrosine, 15 minutes is unnecessarily prolonged for the heating period for the preliminary production of the mercury compound. ⸤That a heating time of 5 minutes is adequate is shown by the colorimetric readings recorded in Table II.⸥

4. In connection with the determination of tyrosine described. above, it might be pointed out that in this case one uses practically the whole of the hydrolysate. It is, therefore, rather more important than in the Folin-Ciocalteu method that no errors are made in

⋆ TABLE II.

*Showing That a 5 Minute Heating Period Is Adequate in the Folin-Ciocalteu Reaction for Tyrosine.*

| Tyrosine. | Heating time. | Colorimetric reading. |
|:---:|:---:|:---:|
| *mg.* | *min.* | |
| 4 | 15 | 20.0 |
| 4 | 10 | 20.0 |
| 4 | 10 | 20.0 |
| 4 | 5 | 19.9 |
| 4 | 5 | 20.0 |
| 4 | 3 | 20.7 |
| 4 | 3 | 20.6 |
| 4 | 2 | 23.0 |

In each experiment the contents of a flask which had been heated for 15 minutes were used as the standard.

following the directions and that the different required solutions are available when the hydrolysate is ready.

The stock solution for all the acids can be a 14 N solution, and a 14 N solution accurate enough for the purpose is obtained by diluting 400 cc. of the pure concentrated acid (sp. gr. 1.82) to a volume of 1 liter. For additional details, such as the purification of tyrosine, of mercuric sulfate, etc., the reader is referred to the Folin-Ciocalteu paper.

5. For the determination of tyrosine in amino acid mixtures obtained by acid hydrolysis, it is essential that the hydrolysis should be made with sulfuric acid as for cystine determinations. Almost every one must know that one cannot apply Millon's reaction for tyrosine in the presence of much chloride, and the

same sort of interference by chlorides is, of course, encountered in the Folin-Ciocalteu reaction. This interference is presumably due to the formation of chlorine. Hydrochloric acid, therefore, cannot be used for the protein hydrolysis.

*Determination of Tryptophane.*—Two slightly different methods were described by Folin and Ciocalteu for the determination of the tryptophane in the centrifuge tube. In this paper we shall describe only the one which seems a little more convenient and which is the one actually used in the analyses reported below.

Add 10 cc. of n HCl to the precipitate in the centrifuge tube and heat in boiling water for 30 minutes. It is quite necessary to heat for this length of time to break up the mercury compound of tryptophane. A trace of insoluble material may remain in the tube; this unknown material does not give a color with the phenol reagent. Cool the heated solution, and filter it through a small filter, accompanied by thorough washing into a 100 cc. volumetric flask. The total volume in the flask should be about 60 cc.

Transfer 1 mg. of tyrosine to another 100 cc. volumetric flask, and dilute to a volume of about 60 cc. Add to each flask 25 cc. of saturated sodium carbonate solution, which has not been in contact with rubber, mix, add 5 cc. of the phenol reagent, described by Folin and Ciocalteu, to each flask, mix again, and let stand for 30 minutes. At the end of this time add 2 or 3 cc. of 5 per cent sodium cyanide to each solution. Dilute to volume, mix, and make the color comparison with the standard, set at 20 mm.

The cyanide does not take part in the reaction, as it is not introduced until after the phenol reagent has been destroyed by the alkali; it is used only to dissolve the precipitated mercury salts.

$1 \times 20$ divided by the colorimeter reading in mm., and the result multiplied by the factor 0.843 gives in mg. the amount of tryptophane present in 20 cc. of hydrolysate. The factor 0.843 represents the color value of tryptophane in terms of tyrosine as was explained in the original paper.

### III. Analyses of Different Proteins by the Micro Method.

The tyrosine and tryptophane values given in this section are not intended to represent revisions of the values reported by Folin and Ciocalteu. The small differences encountered may be due, to a certain extent, to some slight variations in the technique as, for

example, the clearing of all the hydrolysates with kaolin. The proteins examined were obtained from Dr. Edwin J. Cohn, unless otherwise stated.

Our first experiments were made with commercial egg albumin. These were intended only to show the dependability of the methods, in the sense of giving substantially the same values in succes-

TABLE III.

*Commercial Egg Albumin.*

| Determination No. | Tyrosine. | Tryptophane. |
|:---:|:---:|:---:|
| | *per cent* | *per cent* |
| 1 | 3.92 | 1.37 |
| 2 | 4.05 | 1.28 |
| 3 | 4.06 | 1.23 |
| 4 | 3.98 | 1.35 |
| 5 | 3.91 | 1.26 |
| 6 | 3.94 | 1.31 |
| Average. ............. | 3.98 | 1.30 |

TABLE IV.

*Crystallized Egg Albumin (Preparation II, 1925).*

| Determination No. | Tyrosine. | Tryptophane. |
|:---:|:---:|:---:|
| | *per cent* | *per cent* |
| 1 | 4.03 | 1.11 |
| 2 | 4.02 | 1.19 |
| 3 | 4.04 | 1.17 |
| Average. ............. | 4.03 | 1.16 |

sive repetitions. The figures obtained are given in Table III. The figures for crystallized albumin are given in Table IV.

Another sample of egg albumin crystallized three times gave the figures shown in Table V.

*Serum Albumin.*—Two samples of this crystallized protein were analyzed, Tables VI and VII.

In the analyses recorded in Table VIII is represented one pair of determinations (the second) where the process failed to give perfect separation between the tyrosine and the tryptophane. The

tyrosine value, 3.74 per cent, is too high and the corresponding tryptophane value, 1.21 per cent, is too low. Those figures, perhaps, should not go into the record, but it has seemed worth

TABLE V.

*Crystallized Egg Albumin (Sample; Ferry-Wyman, 1928).*

| Determination No. | Tyrosine. | Tryptophane. |
|---|---|---|
| | per cent | per cent |
| 1 | 3.90 | 1.09 |
| 2 | 3.88 | 1.24 |
| 3 | 3.94 | 1.23 |
| 4 | 3.99 | 1.24 |
| Average.............. | 3.93 | 1.20 |

TABLE VI.

*Twice Crystallized Serum Albumin (Sample; Wyman, November,*

| Determination No. | Tyrosine. | Tryptophane. |
|---|---|---|
| | per cent | per cent |
| 1 | 4.51 | 0.52 |
| 2 | 4.66 | 0.54 |
| 3 | 4.66 | 0.52 |
| 4 | 4.81 | 0.54 |
| Average.............. | 4.66 | 0.53 |

TABLE VII.

*Three Times Crystallized Serum Albumin (Sample; Ferry, March, 1927).*

| Determination No. | Tyrosine. | Tryptophane. |
|---|---|---|
| | per cent | per cent |
| 1 | 4.75 | 0.51 |
| 2 | 4.62 | 0.51 |
| 3 | 4.66 | 0.54 |
| Average.............. | 4.67 | 0.52 |

while to show how such errors can be discovered and eliminated. Out of scores of analyses, this error is the only one found where we failed to obtain seemingly complete separation of tryptophane from the tyrosine.

*Muscle Globulin.*—This protein probably has not been analyzed before for tryptophane and tyrosine. It may be worth recording that the need for a micro method came to one of us in connection with a request from Dr. Cohn for an analysis of a sample of muscle

TABLE VIII.

*Cottonseed Globulin from Dr. Osborne's Laboratory.*

| Determination No. | Tyrosine. | Tryptophane. |
|:---:|:---:|:---:|
| | *per cent* | *per cent* |
| 1 | 3.68 | 1.44 |
| 2 | 3.74 | 1.21 |
| 3 | 3.55 | 1.39 |
| 4 | 3.59 | 1.33 |

TABLE IX.

*Muscle Globulin, Not Entirely Pure (Sample; Edsall).*

| Determination No. | Tyrosine. | Tryptophane. |
|:---:|:---:|:---:|
| | *per cent* | *per cent* |
| 1 | 3.93 | 1.55 |
| 2 | 3.91 | 1.57 |

TABLE X.

*Highly Purified Muscle Globulin (Sample; Edsall).*

| Determination No. | Tyrosine. | Tryptophane. |
|:---:|:---:|:---:|
| | *per cent* | *per cent* |
| 1 | 3.92 | 0.98 |
| 2 | 4.00 | 1.03 |
| 3 | 3.96 | 0.97 |
| 4 | 3.82 | 0.99 |
| Average. .. .......... | 3.92 | 0.98 |

globulin. The sample submitted in the form of suspension proved quite inadequate for the regular macro method.

The first small sample submitted probably was not very pure. It gave the values recorded in Table IX, while the purified sample gave the figures recorded in Table X.

*Gliadin.*—We have no less than twenty separate pairs of tyrosine and tryptophane determinations on six different preparations

TABLE XI.

*Gliadin. Six Different Samples.*

|  | Tyrosine. | Tryptophane. |
|---|---|---|
|  | *per cent* | *per cent* |
| Osborne Sample 1 | 3.39 | 0.84 |
| "       "       2 | 3.29 | 0.83 |
| Dill    "       1 | 3.37 | 0.83 |
| "       "       2 | 2.97 | 0.74 |
| Cohn    "       1 c | 3.21 | 0.73 |
| "       "       2 b | 3.27 | 0.78 |

TABLE XII.

*Edestin. Sample; Osborne (6.9 Per Cent Moisture).*

| Determination No. | Tyrosine. | Tryptophane. |
|---|---|---|
|  | *per cent* | *per cent* |
| 1 | 4.13 | 1.34 |
| 2 | 4.01 | 1.32 |
| 3 | 3.96 | 1.44 |
| 4 | 3.91 | 1.38 |
| Corrected average. ... | 4.28 | 1.46 |

TABLE XIII.

*Hempseed Edestin, Not Crystallized (3.8 Per Cent Moisture).*

| Determination No. | Tyrosine. | Tryptophane. |
|---|---|---|
|  | *per cent* | *per cent* |
| 1 | 4.47 | 1.44 |
| 2 | 4.43 | 1.40 |
| 3 | 4.32 | 1.46 |
| 4 | 4.32 | 1.44 |
| 5 | 4.43 | 1.34 |
| 6 | 4.34 | 1.34 |
| Corrected average. ... | 4.54 | 1.45 |

The corresponding figures reported by Folin and Ciocalteu are 4.50 and 1.50.

of gliadin. The repetitions gave perfectly uniform results. It, therefore, seems superfluous to record the individual analyses,

and we give only the averages in Table XI.   Folin and Ciocalteu obtained an average of 3.1 per cent of tyrosine and 0.84 per cent of tryptophane.   We have no explanation to offer for the rather large discrepancy between the tyrosine figures of Folin and Ciocalteu, and those now obtained.   Such differences, of course, could occur if one or the other pair of investigators had used a

TABLE XIV.

*Hemoglobin.   Crystallized Sample.*

| Determination No | Tyrosine. | Tryptophane. |
|---|---|---|
| | per cent | per cent |
| 1 | 3.08 | 1.27 |
| 2 | 3.18 | 1.30 |
| 3 | 3.17 | 1.28 |
| 4 | 3.20 | 1.27 |
| 5 | 3.11 | 1.26 |
| Average. ............. | 3.15 | 1.28 |

TABLE XV.

*Zein.*

| Determination No. | Tyrosine. | Tryptophane. |
|---|---|---|
| | per cent | per cent |
| 1 | 5.80 | 0.19 |
| 2 | 5.89 | 0.20 |
| 3 | 5.88 | 0.20 |
| 4 | 5.94 | 0.20 |
| Average............ .. | 5.88 | 0.20 |

slightly incorrect standard solution of tyrosine, but we are reasonably certain that this has not been the case.

The analytical figures for edestin and for hemoglobin are recorded in Tables XII to XIV.

For the tryptophane determination in zein, Table XV, half quantities of the reagents were taken and the final volume was 50 cc., just as in the original method.

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# AN IMPROVED COLORIMETRIC METHOD FOR THE DETERMINATION OF CYSTINE IN PROTEINS.

BY OTTO FOLIN AND A. D. MARENZI.*

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The colorimetric method of Folin and Looney for the determination of cystine in protein hydrolysates was published in 1922. This method is based on the fact that cysteine is the only known amino acid which reduces the uric acid reagent of Folin and Denis. The conversion of cystine into cysteine is obtained by the reducing action of sodium sulfite. This method has been used by many investigators. It has been criticized more or less, but has not been improved by any of the critics. In the meantime, accurate cystine determinations have become increasingly important. Cystine is a so called essential amino acid, it is present in insulin and in glutathione, and if the cystine determinations are dependable, they supply one important basis for the calculation of molecular weights of proteins. It therefore seemed to us well worth while to reexamine the validity of the Folin-Looney method for the determination of this amino acid.

The Folin-Looney method, as it stands in the literature, has two known defects. It does not provide for the removal of molybdate (and phenol reagent) from the uric acid reagent, in consequence of which one cannot be quite sure that some tyrosine is not included in the cystine determinations. Also, and for the same reason, the reagent gives an uncomfortably large blank with the sodium sulfite which is used for the preliminary reduction of cystine to cysteine. A third defect, discovered in the course of this work, is that cysteine does not react with the uric acid reagent as rapidly as does uric acid. To obtain practically complete reaction with the cysteine it is therefore necessary to add much more of the uric acid reagent than was employed by Folin and

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Looney.  This last defect could scarcely be found and certainly could not be remedied until we had learned how to prepare uric acid reagents entirely free from phenol reagent.  The preparation of this reagent is described in the following paper.

With this phosphotungstic acid reagent the influence of tyrosine is entirely eliminated.  It was further found that Folin and Looney did not use the sodium sulfite to the best advantage.  By adding the sulfite to the acid cystine solution, that is before instead of after the carbonate, the cystine is practically instantly reduced to cysteine even when a relatively small excess of sulfite is added. We were thus able to cut down the amount of 20 per cent sulfite used from 10 cc. to 2 cc., and we have thereby cut down the blank produced by the sulfite until it has become quite negligible.  On the basis of these improvements, it seemed reasonable to expect a better range of true proportionality between colors obtained from different amounts of cystine than could be obtained by the original method.  While this expectation was substantiated, the proportionality obtained seemed to us still not entirely satisfactory.

It is possible to make excellent colorimetric determinations when the range of substantially correct proportionality is limited to colorimetric readings of 14 mm. to 30 mm., when the standard is set at 20 mm., but wherever it is possible to obtain a true range of proportionality between 10 mm. and 40 mm., when the standard is set at 20 mm., one certainly has a much better method.  In the course of our endeavor to attain this range with pure cystine solutions, it was found that the blue color of the reduced uric acid reagent was bleached more or less during the dilution, presumably by the oxygen dissolved in the water.  This source of error is probably a consequence of our using so little sulfite for the reduction of cystine to cysteine.  At all events, by diluting the blue solutions with 3 per cent sodium sulfite solutions instead of water, the bleaching effect of the dilution disappears.  True proportionality between 2 mg. of cystine and 1 mg. or 4 mg. is obtained.  The bleaching effect produced by diluting with water is more noticeable, of course, when one works with small amounts of cystine. With the new process, 0.5 mg. of cystine can be accurately determined by means of the 1 mg. standard, whereas even the 1 mg.

standard loses about 10 per cent of color, if the dilution, to 100 cc., is made with water.

The sum total of all of these refinements of the Folin-Looney method for the determination of cystine is so great that there can scarcely be any comparison in the dependability of the results obtained by the two forms of the method, yet each change represents only a minor improvement, such as one would expect to come in the development of any new method.

For this preliminary control work, as well as for actual determinations in protein hydrolysates, the following reagents are employed:

1. Uric acid reagent free from phenol reagent.

2. A 20 per cent solution of lithium sulfate.

3. A freshly prepared 20 per cent solution of (Merck's) sodium sulfite.

4. A freshly prepared 3 per cent solution of sodium sulfite.

5. A 20 per cent solution of $Na_2CO_3$.

6. A standard cystine solution in N $H_2SO_4$, containing 1 mg. of cystine per cc.

To determine the range of proportionality of the reaction, proceed as follows: Transfer 1, 2, and 4 cc. of the standard cystine solution to 100 cc. volumetric flasks. Add 2 cc. of 20 per cent sodium sulfite solution to each flask. After 1 minute, add also 18 cc. of 20 per cent $Na_2CO_3$ solution and 2 cc. of 20 per cent lithium sulfate solution. Add finally, with shaking, 8 cc. of the uric acid reagent to each flask and let stand for 3 to 4 minutes. Then dilute to volume with 3 per cent sodium sulfite, mix, and make the color comparison. The colorimetric readings will be 40 mm. and 10 mm., when the standard containing 2 mg. is set at 20 mm.

*Determination.*—Transfer from 1 to 5 gm. of dried and accurately weighed protein to a 300 cc. Kjeldahl flask, add 20 cc. of 6 N sulfuric acid and 2 cc. of butyl alcohol (to prevent foaming). Connect with a small vertical condenser and boil gently on a sand bath for 18 to 20 hours. Remove the condenser and boil off the butyl alcohol.

Hydrolysates obtained with acids are less suitable for colorimetric estimations than are alkaline hydrolysates, because of the sometimes large amounts of humin materials which are produced by acids. This trouble is particularly noticeable with proteins

which, like egg albumin, contain carbohydrate groups, or with imperfectly purified casein which probably is not entirely free from lactose. We have made a great many attempts to decolorize the hydrolysates, but the only helpful reagent which we have found is kaolin. All of the others, various kinds of charcoal, Lloyd's reagent, colloidal iron, and lead acetate, adsorb more or less cystine, hence cannot be used although some of them are better decolorizing reagents than kaolin. The best decolorizing agent is probably the charcoal sold under the trade name norit, but norit takes out very much of the cystine and this cystine cannot be washed out.

After the butyl alcohol has been removed, dilute the hydroly-sate in a volumetric flask to 100 cc. and mix. Transfer 2 gm. of kaolin to a 200 cc. flask, add the hydrolysate, shake gently for 3 to 5 minutes, and filter. If desired, the kaolin can be added to the undiluted hydrolysate in the Kjeldahl flask, followed by filtration and thorough washing and dilution to a volume of 100 cc.

From 1 to 5 cc. of the nearly decolorized hydrolysate are taken for the determination.

Transfer the required amount to a 100 cc. volumetric flask, and 2 cc. of the standard cystine solution to another. Add 2 cc. of freshly prepared 20 per cent solution of sodium sulfite (Merck) to each flask and let stand for 1 minute. Then add 18 cc. of the sodium carbonate solution to the standard, and to the unknown add 18 cc. plus 0.5 cc. for each cc. by which the hydrolysate taken exceeds 2 cc. For example, if 4 cc. of hydrolysate were taken, use 19 cc. of carbonate solution. No great accuracy is needed in the addition of the carbonate; it is done with a measuring cylinder, but we found by careful experiments that 18 cc. of 20 per cent carbonate are best for 2 cc. of standard solution of cystine in normal acid, and the directions as given are merely intended to correspond to that observation. After the addition of the car-bonate, add also 2 cc. of 20 per cent lithium sulfate solution, and finally add, with shaking, 8 cc. of uric acid reagent. Let stand for 3 to 5 minutes, dilute to volume, and make the color comparison.

## Cystine Content of Proteins.

*1. Casein.*—In casein prepared according to Hammarsten, an old Kahlbaum sample, we found 0.22 per cent of cystine. But in

a sample of casein purified in Cohn's laboratory (by Pertzoff) we obtained the following figures: 0.31, 0.30, 0.29, 0.30.    Average 0.30 per cent of cystine.

This is a higher figure for the cystine content of casein than other investigators have found; Mörner (1902) found only 0.1 per cent.

*2. Gliadin.*—Two samples of gliadin were analyzed (Dill No. 4 and Dill No. 2 B).    The first sample gave values of 2.07, 2.06, and 2.06; average 2.06 per cent.    These values when corrected for 6.67 per cent of moisture gave an average value of 2.19 per cent of cystine.    The second gliadin contained 6.7 per cent of moisture, and gave as the corrected average value 2.175 per cent of cystine.

Folin and Looney found 2.32 per cent of cystine in gliadin. Osborne and Clapp, by gravimetric determinations (1906), found only 0.5 per cent.    Van Slyke (1912) found 1.17 per cent, by his method.

*3. Edestin.*—Three different samples of edestin were analyzed, but the first was available in such a small amount that we could not determine the moisture content.    It gave an uncorrected value of 1.24 per cent cystine.

The second sample (labelled Wakeman), after drying at 110°, gave 1.36, 1.35, 1.40; average 1.37 per cent cystine.

The third sample, a crystallized product from Dr. Osborne's laboratory, gave us 1.24, 1.27, 1.27; average 1.27 per cent, and after correction for moisture, this sample gave an average cystine value of 1.35 per cent.

The original Folin-Looney method had yielded only 0.75 per cent and 0.84 per cent of cystine, and Abderhalden found only 0.3 per cent of cystine in edestin.

*4. Zein.*—Our sample of zein obtained like our other proteins from Dr. Cohn, was labelled (Nolan).    It gave the following cystine values: 0.98, 0.97, 0.98, 0.98 per cent.    It contained 5.54 per cent of moisture and thus gave us a final corrected average cystine value of 1.03 per cent.

Folin and Looney had found only 0.5 per cent and, later, Looney  · found 0.75 per cent.

*5. Egg Albumin.*—Two samples of this protein were obtained from Dr. Cohn.    The first labelled R. M. F., 1927, was dried at 110° and gave the following cystine values: 1.23, 1.22, 1.22;

average 1.22 per cent. The second sample labelled Ferry and Wyman gave these values for the dried material: 1.28, 1.20, 1.23, 1.20; average 1.23 per cent. These two samples of egg albumin had been recrystallized four and three times respectively.

The original Folin-Looney process had given only 0.81 per cent as the cystine content of egg albumin.

*6. Serum Albumin.*—Two different samples, both recrystallized four times were analyzed. The sample labelled Ferry gave these values on the dried material: 6.00, 6.05, 6.02 per cent; average 6.02 per cent of cystine. The sample labelled Wyman gave 6.09, 6.09, 6.04, 6.04, 6.04; average 6.06 per cent of cystine.

Some brief comments on these cystine values obtained on purified protein materials may be permitted.

When these analyses were begun we had no idea as to how they might compare with figures previously published in the name of the Folin-Looney method, but the differences encountered are larger than we expected. Only in the case of gliadin are the new figures (a little) lower than the figures obtained by the original method. In all of the others, they are higher, often very much higher. Those who are antagonistic to colorimetric methods will find material for general criticisms of colorimetric methods in these large discrepancies. But it may be pointed out that the new analytical procedure represents several tangible improvements in the method, improvements whose importance is demonstrable by work on pure cystine solutions. Furthermore, large as these differences are, larger differences still are encountered by comparing the gravimetric analyses with our colorimetric results. Thus in casein, Mörner found 0.1 per cent of cystine where we find 0.3 per cent; in gliadin, Osborne found 0.5 per cent where our figure is 2.19 per cent; and in edestin, Abderhalden found 0.3 per cent against our value of 1.36 per cent. Gravimetric analyses, even in the hands of such investigators as those mentioned, have given practically meaningless results.

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# THE PREPARATION OF URIC ACID REAGENT COMPLETELY FREE FROM PHENOL REAGENT.

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It had been known for a long time that ordinary phosphotungstic acid in alkaline solutions gives some blue color with certain reducing substances when, in 1912, it was shown by Folin and Denis that this property of phosphotungstic acid could be increased enormously by the introduction of suitable changes in the mode of preparing the solutions. The solution thus obtained from sodium tungstate and phosphoric acid was introduced as a uric acid reagent, while the solution produced by the action of phosphoric acid on sodium tungstate plus sodium molybdate became their phenol reagent. A systematic study of the subject, by Wu (1) in 1920, showed that the uric acid reagent is phospho-18-tungstic acid, whereas ordinary phosphotungstic acid consists almost entirely of the inactive phospho-24-tungstic acid. Many variations and some improvements in the preparation of the uric acid reagent have been described since 1912. But, while it was recognized from the beginning that the greatest attainable degree of specificity could be obtained only in the absence of molybdate, it was not until 1924 that the first attempt was made, by Folin and Trimble (2), to provide for the removal of the molybdates which are present in varying amounts in all available samples of sodium tungstate. Folin and Trimble described a very satisfactory test for the molybdates and also gave a method for their partial removal by means of $H_2S$, as a preliminary step in the preparation of the uric acid reagent. Manufacturers of sodium tungstate have in the meantime contributed nothing except to meet the demand for normal tungstates as distinguished from the

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less soluble $p$-tungstates.   The brands now available, except
Merck's best "reagent" tungstate, seem to contain even more
of molybdates than was the case a few years ago.

'Scheiner (3) an Italian, has recently come to the conclusion that there is
practically very little difference between the phenol reagent and the uric
acid reagent   Scheiner's paper deals exclusively with the alleged useless-
ness of the reagents, and he does not state what particular determination
he was trying to learn.   The title of his paper may be given as "Sources
of Error in the Determination of Phenols, Tyrosine and Uric Acid by
means of Phosphotungstic Acid [Uric Acid Reagent]."

One of the most exacting needs for a uric acid reagent which
is free from phenol reagent is encountered in attempting to
make cystine determinations in protein hydrolysates accord-
ing to the method of Folin and Looney.   To get a reagent
which gives the maximum color with cysteine, yet gives no
trace of color with tyrosine, and a negligible color with the
sodium sulfite which must be used for reducing cystine to
cysteine, it is quite essential that a reagent with the highest
degree of selectivity be secured.   Indeed it might be fairly
questioned whether one is not here asking for too much in the
way of colorimetry based on selective oxidation.

In the course of a proposed critical study of the colorimetric
cystine determination, we found that the uric acid reagents
prepared according to Folin and Trimble from the sodium tung-
states now available could not be made to yield usable results.
One old uric acid reagent made by one of us 4 years ago was
very nearly satisfactory, but we were not able to reproduce it.
In these circumstances we were led to revise the Folin-Trimble
process and finally succeeded in developing a method which,
from all brands of sodium tungstate now available, gives uric
acid reagents quite free from phenol reagent.   Our method is
based on the following principles and observations:

1. Sodium molybdate does, sodium tungstate does not, com-
bine with phosphoric acid at room temperatures.

2. After the addition of the right amount of phosphoric acid
to a sodium tungstate solution, the uncombined molybdate reacts
fairly rapidly with $H_2S$.

3. Most of the molybdenum is converted into brown insoluble

sulfides by the H₂S treatment, and these sulfides are removed at once by filtration.

4. A part of the molybdate is converted into soluble sulfur compounds by the $H_2S$ treatment, and the longer this treatment is continued the greater will be the fraction of soluble molybdenum sulfur compounds.

5. The soluble sulfomolybdates are very soluble in alcohol, and can be quantitatively removed by a single 5 minute extraction with alcohol.

On the basis of these principles and observations 100 gm. of sodium tungstate can now be converted in the course of about 2 hours into 1 liter of uric acid reagent completely free from phenol reagent.

The process is as follows:

Transfer 100 gm. of sodium tungstate and 200 cc. of water to a 500 cc. Florence flask. Shake until the tungstate is dissolved. Add slowly, with shaking and cooling, 20 cc. of 85 per cent phosphoric acid. The solution must not be allowed to become warm from the heat of the reaction with the phosphoric acid. Pass $H_2S$ into the phosphotungstate solution at a very moderate rate for 20 minutes. At the end of the first 3 or 4 minutes, add gradually and slowly another 10 cc. of 85 per cent phosphoric acid without interrupting the $H_2S$ current. It would be simpler to add all of the phosphoric acid (30 cc.) before beginning the $H_2S$ treatment, but by adding the phosphoric acid as described, one obtains the molybdenum sulfide in a somewhat less finely divided condition, so that it can be removed more easily by filtration. Incidentally, it may be remarked that the 30 cc. of phosphoric acid should be just sufficient to render the solution slightly acid to Congo red paper. At the end of 20 minutes, filter the solution through a good grade of quantitative filter paper. It is advisable to collect the first 40 cc. of filtrate in a 50 cc. cylinder, because the first portion may be a little turbid, and it may need to pass through the filter a second time.

If the conditions have been right, the filtrate should be clear, and it will have a greenish color, because a little blue is produced by reduction of any uric acid reagent that may have formed, while the soluble sulfomolybdates are red.

Transfer the filtrate to a separatory funnel (capacity 1 liter) and add, with shaking, 300 cc. (1.5 volumes) of alcohol. The mixture separates at once into a reddish or slightly greenish supernatant solution, and a bluish, very heavy solution at the bottom. The latter contains all of the phosphotungstic acid in a supersaturated solution, and it is best to withdraw it rather soon into a *weighed* 500 cc. Florence flask. If left too long in the separatory funnel, it sometimes forms crystal deposits which block the exit through the stop-cock.

In so far as any insoluble molybdenum sulfide happens to be present, this will be floating between the two layers of liquid in the separatory funnel, and these solid aggregates must not be allowed to pass through the stop-cock and into the phosphotungstic acid solution. The mixture remaining in the separatory funnel is discarded. It contains not only the sulfomolybdates, and the greater part of the surplus $H_2S$, but probably also various other impurities.

Add water to the concentrated phosphotungstic acid in the 500 cc. flask until the weight of the contents amounts to 300 gm. Boil the solution over a micro burner for a few minutes, until a paper moistened with lead acetate solution shows that the $H_2S$ has been removed. Then, but not until then, cut down the flame, and add 20 cc. of 85 per cent phosphoric acid. It is only with the addition of this last quantity of phosphoric acid that the optimum conditions are obtained for transforming the ordinary (1:24) phosphotungstic acid into the active (1:18) phosphotungstic acid, that is to say, into the uric acid reagent.

Insert a 10 cm. funnel into the 500 cc. flask to hold a 200 cc. flask filled with cold water, and boil gently for 1 hour. At the end of this time, the reaction is finished. Cut down the flame, remove the condenser (funnel and flask), filter, and add to the filtrate a few drops of bromine, and boil, to remove the blue color of the solution. When the blue color is gone, boil rapidly for a few minutes, to remove the bromine, then cover the mouth of the flask with a beaker and cool under running water.

Transfer 25 gm. of lithium carbonate to a liter beaker, add first 50 cc. of phosphoric acid, then add slowly 250 cc. of water and boil, to remove the $CO_2$. Cool the resulting lithium phos-

phate solution; and add it to the concentrated uric acid reagent in the 500 cc. flask and dilute to 1 liter.

The reagent which we obtain by the process described above is better than any uric acid reagent we have ever had before in this laboratory, not only for cystine determinations, but also for uric acid determinations. It is very active, yet possesses the highest obtainable degree of specificity, hence, gives the minimum of blanks, with sulfite, cyanide, etc., and does not give a trace of color with tyrosine. In addition, it shows less tendency to give disturbing precipitates than do our other uric acid reagents.

The blue color which this reagent gives with uric acid is of a different shade from the blue which is obtained with uric acid reagents which have been prepared in the ordinary way, that is, without the preliminary removal of the molybdate. The blue from the ordinary reagents has, by comparison, a distinctly violet tint. This point is rather suggestive in connection with Wu's detailed work on the active ingredients of the uric acid reagent. By fractional crystallization, Wu obtained two active compounds which he called the A and the B form of phospho-18-tungstic acid. These differed in chromogenic values, and they differed markedly in the shade of blue which they produced with uric acid. It seems altogether probable that the finding of these two different compounds represents the fact that the sodium tungstate from which they were prepared contained sodium molybdate.

While convenience and speed of preparation were considered of minor importance in developing the method described above, the process is so simple that all who have occasion to prepare and use the uric acid reagent should be able and willing to first remove the molybdate—until such time as some manufacturers can be induced to supply sodium tungstate which is completely free from molybdate.

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# AN IMPROVED FORM OF FOLIN'S MICRO METHOD
# FOR BLOOD SUGAR DETERMINATIONS.

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Some time ago a supplementary note on Folin's micro method for blood sugar determination was published. This note was deemed indispensable because of the unfortunate recommendation of toluene as a preservative for the dilute tungstic acid reagent. Incidentally, certain other improvements were given, chief of which was the use of gum ghatti instead of gum arabic as a protective colloid. It can now be stated that the gum ghatti solutions keep practically indefinitely. This statement is based on the observation that the solution has kept in seemingly perfect condition for over 5 months in an incubator, 37°. A more thorough revision and improvement of the method is given in this paper.

The micro method as given in the original paper and supplement is quite satisfactory for all kinds of blood—except those encountered in diabetic clinics. These bloods represent enormous variations; they may contain anywhere from under 50 to over 600 mg. per cent of sugar, and it is often important that the analyses be finished as quickly as possible. The range of the micro method, 50 to 200 mg. per cent, is rather limited when considered in relation to such bloods, for it is only by judicious repetitions of the determination with varying amounts of blood extract that one can be sure to cover the amount present on the basis of the limited quantity of extract (9 cc.) obtainable from 0.1 cc. of blood.

Our efforts to enlarge the range of the method so as more nearly to meet the needs of diabetic clinics have been more successful than was at first thought possible. We have in fact secured a

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much wider dependable range than is obtainable by any other colorimetric method for blood sugar and, at the same time, we have made the method distinctly easier than it was before.

The problem involved was only that of how to measure Prussian blue in the presence of a large surplus of the yellow ferricyanide, because the underlying reaction—the oxidation of sugar by the ferricyanide—is far more efficient than is the oxidation of sugar with alkaline copper solutions.

It was soon found that the disturbing effects of the yellow ferricyanide could be removed by easily constructed light screens. Filter paper stained a deep yellow with picric acid makes a perfectly adequate light filter for the purpose   When the opening of an ordinary colorimetric light box is covered with a piece of such paper, one can use 4 times as much ferricyanide as in the original method, and with one standard and one determination can cover the range between 25 mg. per cent and 400 mg. per cent of blood sugar.   In fact, one can get approximate valuations of blood sugars as high as 700 or 800 mg. per cent.   Nor is there any need for balancing the yellow colors of the standard and the unknown by any second addition of ferricyanide.

When such light filters are used, the method becomes rather more photometric than colorimetric, for the blue color is mostly replaced by light absorption, and the end-point in the colorimeter adjustment is more or less like what one observes in a polariscope. The two fields remain different in two respects, namely intensity of light and quality of color until the point of equality is reached. These comparisons seem to us easier on the eye and easier to make with certainty than the usual color comparisons where the quality of color remains uniform and the only variable is the intensity.

The principle of using selective light screens or selected light rays for photometric and colorimetric comparisons is, of course, far from new, but in work with the modern colorimeters the aim heretofore has been to secure a light corresponding to diffuse daylight (by the use of "daylite" glass).   And because of this practice we have been rather sharply confined to the use of chromophoric reagents which have no color of their own or which at least do not have a quite different color from that which is to serve as a basis for comparison.   The success which we have obtained with the picric acid light filters leads us to believe that

by the help of suitable light filters (in the form of colored paper or in the form of solutions) the scope of practical colorimetry may be greatly enlarged.

### Preparation of Acid Picrate Light Filter.

Dissolve 5 gm. of picric acid in 100 cc. of methyl alcohol and add 5 cc. of 10 per cent sodium hydroxide solution. Place a pack of eight to ten filter papers on a level and smooth mat of newspapers. Pour the acid picrate solution onto the filters until the papers are saturated and an excess of solution filters through at the bottom and flows out a distance of at least 2 cm. on the newspaper mat. When all the liquid has evaporated and the filter papers are perfectly dry, pour over the pack an excess of a 3 per cent solution of paraffin in benzine (gasoline) and again leave the papers to dry. All of the filter papers will be evenly stained, canary yellow to golden yellow, and the stain will not rub off, because of the paraffin. A heavy filter with good absorbing qualities, such as Schleicher and Schüll, No. 604, is best. Large or small filters stain equally well.

The size of papers taken will be determined by the window in the light box. There is no uniformity in these arrangements, as there are various different kinds of light boxes, and many people use home made ones. The best lamp for colorimeter work is the Chalet microscope lamp (Eimer and Amend Catalogue No. 27910) with 150 watt gas-filled Mazda bulb and one 82 mm. × 82 mm. "daylite" glass window. With a 150 watt light bulb this lamp gives a very strong light, and a strong light is almost essential when picrate light filters are used. For this reason it is probably best to use the mirror side of the colorimeter reflector for these sugar determinations, while for practically all other determinations the dull faced reflector is best. Important points of merit in the Chalet lamp are its compactness and the absence of an attached platform for the colorimeter to stand on. No such platform is desirable, although our home-made light boxes have them. The best platform for the colorimeter to stand on is a piece of heavy plate glass polished on one side and preferably rough on the other side. When standing on such a support, the colorimeter, which is rather heavy, is easily moved into the right position, and this must be done for the preliminary color comparison

with the standard.  No platform arrangement which tends to
interfere with adjustments in the position of the colorimeter with
reference to the light should be used.

With the Chalet lamp or most home-made light boxes, suitable
picrate light filters are obtained from filters having a diameter
of 12.5 cm.  The yellow paper is pasted with adhesive plaster
over the "daylite" window and if desired the "daylite" glass may be
removed.  In order to facilitate the use of the colorimeter for all
other purposes it is, however, best to keep the light filter attached
to a suitable frame so that it can be put in place or removed
instantly.  These light filters attached to a strong, yet light,
compressed cork frame, with extra filters, can be obtained from
Eimer and Amend.  Our own frames are made from compressed
cork rings, with an internal diameter of 3.5 inches.  With the
help of a wood file, it is only a few minutes work to convert such
a cork ring into a suitable holder for the light filter.  A handle is
made from a cork and a screw.  One advantage of these frames is
that, if it should become desirable, one can tack a light filter on
each side,—if the illumination is strong enough, as it is on the
Chalet lamp.

*Test of Adequacy of Light Filter.*—Half fill one colorimeter cup
with 0.2 per cent potassium ferricyanide solution and half fill the
other cup with water.  Set both plungers at 20 mm., and make
sure that there are no air bubbles under the plungers.  With the
light filter in place, adjust the colorimeter so that the two fields
look alike.  If the light filter is inadequate the equality of the
two fields cannot be obtained.

### Description of Revised Method.

The following reagents are used:

*1. Dilute Tungstic Acid Solution.*—Transfer 20 cc. of 10 per
cent sodium tungstate to a volumetric liter flask.  Dilute to a
volume of 800 cc., add with shaking 20 cc. of $\frac{2}{3}$ N sulfuric acid, and
dilute to volume.

*2. Potassium Ferricyanide Solution.*—Dissolve 2 gm. of C.P.
potassium ferricyanide in distilled water and dilute to a volume of
500 cc.  The major part of this solution should be kept in a
brown bottle in a dark closet.  The reagent in daily use should
also be kept in a brown bottle.

*3. Sodium Cyanide-Carbonate Solution.*—Transfer 8 gm. of anhydrous sodium carbonate to a 500 cc. volumetric flask. Add 40 to 50 cc. of water and shake, to promote rapid solution. With a cylinder, add 150 cc. of freshly prepared sodium cyanide solution, 1 per cent; dilute to volume and mix.

*4. Ferric Iron Solution.*—Fill a liter cylinder with water. Suspend on a copper wire screen, just below the surface, 20 gm. of soluble gum ghatti, and leave overnight (18 hours). Remove the screen, and strain the liquid through a double layer of a clean towel. Add to this extract a solution of 5 gm. of anhydrous ferric sulfate in 75 cc. of 85 per cent phosphoric acid plus 100 cc. of water. Add to the mixture, a little at a time, about 15 cc. of 1 per cent potassium permanganate solution to destroy certain reducing materials present in gum ghatti. The slight turbidity of the solution will disappear completely, if kept at 37° for a few days.

*5. Standard Glucose Solution.*—The working standard is made to contain 0.01 mg. of c.p. glucose per cc.

*Determination.*—With an accurate 0.1 cc. pipette, described in the original paper, collect 0.1 cc. of blood and transfer it to 10 cc. of dilute tungstic acid in a centrifuge tube. Stir well and centrifuge. Transfer 4 cc. of the water-clear supernatant extract to a test-tube graduated (with a ring going all around) at 25 cc. Transfer 4 cc. of the standard sugar solution to another similar tube. To each tube add 2 cc. of the 0.4 per cent potassium ferricyanide solution and 1 cc. of the cyanide-carbonate solution. Heat in boiling water for 8 minutes and cool in running water for 1 to 2 minutes. Add 5 cc. of the ferric iron solution and mix. Let stand for 1 to 2 minutes, and then dilute with water nearly, but not quite, to the 25 cc. mark. The surface of the solutions is apt to be obscured by the presence of a little foam. Destroy this by the addition of a couple of drops of alcohol. Then dilute exactly to the 25 cc. mark and mix.

Half fill the colorimeter cups with the green-colored standard, set the two plungers at a height of 20 mm., and cover the opening of the light box with the picric acid light filter. Adjust the position of the colorimeter and of the mirror glass reflector until the two fields look exactly alike. If this cannot be done

readily the colorimeter is probably defective. For this adjustment it is helpful to keep the colorimeter standing on a piece of polished plate glass.

Rinse one colorimeter cup and plunger with the unknown solution, and pour the unknown into the cup to a suitable height. When the unknown is very dark, as when one is working with diabetic bloods, a depth of 1 cm. for the unknown is usually ample; in cases of hypoglycemia, the cup needs to be more than half filled. The final color comparison is then made in the usual manner.

Colorimeter readings between 40 mm. and 5 mm. may be accepted as correct and dependable provided that perfect equality as to light and color is obtained.

A little explanation of the last remark may prove helpful. For some time after we had perfected the use of the light filter we were not able to secure correct values for very strong sugar solutions, and it soon became apparent that the discrepancy . was due to an inequality of light rather than of color. The field representing the weaker solution always seemed a little more opaque or less transparent than the other. It is not yet entirely clear to us how this inequality in light is produced, but the trouble disappeared when we increased the amount of ferric iron solution from 3 cc. to 5 cc. By this increase we incidentally also eliminated the need of a 5 minute waiting period for the development of the color. The maximum color is obtained almost instantly.

# BLOOD SUGAR AND FERMENTABLE BLOOD SUGAR AS DETERMINED BY DIFFERENT METHODS.

By OTTO FOLIN and HAQVIN MALMROS.*

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## I. Comparison between Four Different Methods for Blood Sugar Determination.

A protracted period of intensive critical study of the three methods for the determination of blood sugar which have been devised in this laboratory, has yielded improvements of undoubted merit in connection with each method (1). One reason for making such a study was the need to subject the findings of Folin and Svedberg (2) to a closer scrutiny. If those findings were erroneous, it could only be because the methods were not sufficiently dependable for that kind of work. As a matter of fact, the defects found in the two copper methods would seem to be serious enough to more or less invalidate the comparative results reported by Folin and Svedberg, and made a repetition of the work with the improved methods imperative.

Quite apart from the possible existence in blood of another sugar than glucose, it seemed necessary to show by actual analyses how much difference it makes in the blood sugar values whether one or another of the revised methods is used. Since the vast majority of blood sugar determinations, made in Europe, are made by the Hagedorn-Jensen method, it seemed desirable to include this method also in our comparisons. Originally we thought that we might want to use the Hagedorn-Jensen method in our attempt to solve the non-glucose sugar problem, but, as the preliminary comparative analyses accumulated, it became evident that this method could supply no additional information, and, as it is more laborious than the other methods, we did not continue with it.

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Notwithstanding the extensive use which has been made of the Hagedorn-Jensen method, it would seem that this process has never been described with sufficient detail to enable moderately critical workers to avoid certain pitfalls which may lead to erroneous results. We have reference here to the filtration used in this method for the removal of the coagulated proteins.

In the Hagedorn-Jensen method, the total blood filtrate is used, and the titration is made on the filtrate plus wash water and here is where errors can creep in. The filtration is made through carefully washed absorbent cotton. It is very important that not a trace of coagulum gets into the filtrate, because otherwise the results obtained will be too high. The obvious way to avoid such an error would be to close the stem of the funnel with a small and sufficiently tight plug of cotton. This is not permissible, however, because even the most thoroughly washed cotton would give off reducing materials to the filtrate under such conditions. A tuft of wet cotton is practically just dropped into the funnel in order to give a sufficiently rapid filtration to avoid getting reducing carbohydrates from the cotton. This process is perfectly good, for the coagulum and zinc carbonate mixture is retained quite perfectly by the loose cotton deposit. But in order to secure the indispensable perfect retention of the coagulum, one naturally would err on the side of taking too much rather than too little cotton. Yet if too much cotton is used, it retains so much mother liquor that the subsequent definitely prescribed washing process does not remove it, and the results will be too low. It is, therefore, necessarily a matter of judgment and experience how to make this filtration so as to get all the sugar and no coagulum in the filtrate, and it is to be feared that a great many sugar determinations made by this process do not truly represent the values which the Hagedorn-Jensen method can give.

The Hagedorn-Jensen method gives perfectly consistent and dependable results, if the method is worked with scrupulous attention to all details, including daily blank determinations. One suggestion in regard to the method might be made. The alkaline ferricyanide reagent does not keep very well, even in the dark, and it would probably be better to keep that reagent in the form of two separate stock solutions.

In Table I are given figures showing that when as much as

TABLE I.

*Hagedorn–Jensen Method.*

| Concentration of glucose solution. | Filtering through small cotton filter.* Found. | Filtering through larger cotton filter.† Found. |
|---|---|---|
| *mg. per cent* | | |
| 100 | 99 | 95 |
| | 99 | 93 |
| | 99 | 88 |
| | 99 | 96 |
| | 98 | 97 |
| | 98 | 97 |
| | 99 | 95 |
| | 100 | 93 |
| | 100 | 93 |
| | 99 | 97 |
| 200 | 201 | 192 |
| | 198 | 195 |
| | 199 | 193 |
| | 200 | 193 |
| | 199 | 193 |
| | 197 | 181 |
| | 199 | 189 |
| | 200 | 192 |
| | 199 | 193 |
| | 199 | 189 |

\* Weight about 20 mg.

† Weight about 100 mg.

TABLE II.

*Hagedorn-Jensen Method.*

| | Small cotton filter. | Error. | Larger cotton filter. | Error. |
|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *mg.* |
| 1. Sugar in original blood......... | 101 | | | |
| Same, plus 51 mg. glucose...... | 151 | −1 | 143 | −9 |
| 2. Sugar in original blood.......... | 82 | | | |
| Same, plus 95 mg. glucose..... | 175 | −2 | 164 | −13 |
| 3. Sugar in original blood......... | 56 | | | |
| Same, plus 96 mg. glucose...... | 150 | −2 | 132 | −20 |
| 4. Sugar in original blood.......... | 94 | | | |
| Same, plus 199 mg. glucose..... | 290 | −3 | 280 | −13 |
| 5. Sugar in original blood......... | 83 | | | |
| Same, plus 200 mg. glucose..... | 282 | −1 | 271 | −12 |

100 mg. of absorbent cotton are used for the filtration, there is always a loss of sugar. In these determinations, standard glucose

TABLE III.
*Blood Sugar in Mg. Per Cent.*

| Determination No. | Folin. (a) | Folin-Wu.* (b) | Difference between (a) and (b) | Folin micro method. (c) | Difference between (a) and (c) | Hage-dorn. (d) | Difference between (a) and (d). |
|---|---|---|---|---|---|---|---|
| 1 | 81 | 93 | +12 | 86 | +5 | 87 | +6 |
| 2 | 86 | 95 | +9 | 90 | +4 | 88 | +2 |
| 3 | 76 | 84 | +8 | 78 | +2 | 79 | +3 |
| 4 | 82 | 96 | +14 | 83 | +1 | 91 | +9 |
| 5 | 79 | 91 | +12 | 90 | +11 | 92 | +13 |
| 6 | 85 | 96 | +11 | 91 | +6 | 91 | +6 |
| 7 | 84 | 90 | +6 | 86 | +2 | 89 | +5 |
| 8 | 76 | 83 | +7 | 78 | +2 | 79 | +3 |
| 9 | 77 | 78 | +1 | 78 | +1 | 77 | ±0 |
| 10 | 75 | 77 | +2 | 77 | +2 | 77 | +2 |
| 11 | 74 | 84 | +10 | 74 | ±0 | 74 | ±0 |
| 12 | 89 | 94 | +5 | 91 | +2 | 94 | +5 |
| 13 | 103 | 114 | +11 | 110 | +7 | 111 | +8 |
| 14 | 125 | 135 | +10 | 133 | +8 | 130 | +5 |
| 15 | 87 | 93 | +6 | 92 | +5 | 89 | +2 |
| 16 | 83 | 96 | +13 | 87 | +4 | 89 | +6 |
| 17 | 86 | 98 | +11 | 89 | +3 | 96 | +10 |
| 18 | 98 | 111 | +13 | 99 | +1 | 107 | +9 |
| 19 | 113 | 130 | +17 | 121 | +8 | 121 | +8 |
| 20 | 127 | 147 | +20 | 151 | +24 | 151 | +24 |
| 21 | 138 | 158 | +20 | 152 | +14 | 156 | +18 |
| 22 | 153 | 170 | +17 | 158 | +5 | 164 | +11 |
| 23 | 154 | 167 | +23 | 159 | +5 | 164 | +10 |
| 24 | 155 | 170 | +15 | 165 | +10 | 170 | +15 |
| 25 | 179 | 212 | +33 | 209 | +30 | 208 | +29 |
| 26 | 181 | 204 | +22 | 190 | +9 | 188 | +7 |
| 27 | 196 | 226 | +30 | 212 | +16 | 209 | +13 |
| 28 | 224 | 244 | +20 | 237 | +13 | 229 | +5 |

\* To get the most accurate values, a table of correction, previously published by Folin, has been used.

solutions were subjected to the Hagedorn-Jensen treatment, and the zinc carbonate was removed by filtration.

In Table II, we give results showing that the recovery of glucose added to blood by the Hagedorn-Jensen method is satisfactory,

if the right cotton filter is used, but it is not good if the filter is too large.

TABLE IV.

*Blood Sugar in Mg. Per Cent.*

| Determina-tion No. | Folin. | Fermentable sugar. | | | | |
|---|---|---|---|---|---|---|
| | | Folin.<br>(a) | Folin-Wu.<br>(b) | Difference<br>between<br>(a) and (b). | Folin micro<br>method.<br>(c) | Difference<br>between<br>(a) and (c). |
| Normal blood. | | | | | | |
| 1 | 56 | 47 | 47 | ±0 | 46 | −1 |
| 2 | 81 | 72 | 73 | +1 | 72 | ±0 |
| 3 | 83 | 74 | 79 | +5 | 77 | +3 |
| 4 | 88 | 80 | 87 | +7 | 82 | +2 |
| 5 | 95 | 86 | 92 | +6 | 89 | +3 |
| 6 | 129 | 120 | 128 | +8 | 121 | +1 |
| Diabetic blood. | | | | | | |
| 7 | 84 | 74 | 78 | +4 | 78 | +4 |
| 8 | 105 | 99 | 103 | +4 | 102 | +3 |
| 9 | 114 | 101 | 109 | +8 | 107 | +6 |
| 10 | 130 | 120 | 122 | +2 | 122 | +2 |
| 11 | 138 | 131 | 130 | −1 | 129 | −2 |
| 12 | 142 | 131 | 145 | +14 | 145 | +14 |
| 13 | 148 · | 136 | 145 | +9 | 144 | +8 |
| 14 | 152 | 141 | 142 | +1 | 147 | +6 |
| 15 | 152 | 142 | 141 | −1 | 142 | ±0 |
| 16 | 162 | 150 | 159 | +9 | 157 | +7 |
| 17 | 163 | 154 | 157 | +3 | 159 | +5 |
| 18 | 167 | 157 | 161 | +4 | 158 | +1 |
| 19 | 169 | 158 | 161 | +3 | 161 | +3 |
| 20 | 174 | 165 | 166 | +1 | 164 | −1 |
| 21 | 185 | 171 | 183 | +12 | 184 | +13 |
| 22 | 195 | 185 | 194 | +9 | 192 | +7 |
| 23 | 220 | 212 | 224 | +12 | 224 | +12 |
| 24 | 225 | 217 | 233 | +16 | 233 | +16 |
| 25 | 225 | 214 | 233 | +19 | 229 | +15 |
| 26 | 298 | 292 | 297 | +5 | 296 | +4 |
| 27 | 323 | 310 | 326 | +16 | 319 | +9 |
| 28 | 332 | 319 | 325 | +6 | 322 | +3 |

In Table III are given the sugar values for twenty-eight different samples of blood by four different methods. From these

figures it is clear (1) that the Folin-Wu method in the revised form still gives definitely higher values than any of the three other methods; (2) the revised copper method of Folin gives the lowest values; (3) the two ferricyanide methods give very nearly identical values. It should be noted that these values are obtained from the same samples of *venous* blood, as were used for the copper method, while in actual practical application one would use capillary blood for these methods. In a few instances the two ferricyanide methods do not agree. These differences are not due to avoidable analytical errors, they were found by repeated determinations to represent real differences, but they are probably due only to the presence of a somewhat too large amount of oxalate or fluoride, by virtue of which the protein precipitation might have been a little short of perfect. Such differences should, therefore, never occur when capillary blood is used. The diabetic bloods were obtained from Dr. Joslin's patients at the Deaconess Hospital, and some of these samples might have contained excessive amounts of sodium fluoride.

At all events, it is fairly clear from the figures given in Table III that nothing was to be gained by continuing to use both ferricyanide methods in connection with our study of the fermentable sugar.

## II. *Fermentable Sugar in Blood.*

Our study of the fermentable sugar was begun only after we had collected the data reported in Tables I to III. In this study, we have followed, with the revised methods, the procedure of Folin and Svedberg, except that we extended the fermentation period to 10 minutes. We also used washed yeast, although we do not consider the preliminary washing of yeast as essential or even important. The non-fermentable rest reductions which we have obtained are substantially identical with those found by Folin and Svedberg by the use of unwashed yeast. The bloods taken for these determinations were collected especially for us from Dr. Joslin's diabetic patients, or by ourselves from normal subjects. Lithium oxalate cloth was used as anticoagulant.

The analytical results obtained from twenty-eight different samples of blood are recorded in Table IV. In twelve of these twenty-eight samples of blood, the fermentable sugar values as

determined by the two copper methods come within 4 mg. per cent of each other, and in two others with 298 and 332 mg. per cent of blood sugar, the differences are only 5 and 6 mg. per cent. All of these might be interpreted as coming within the limits of experimental error.

The other fourteen samples, recorded in Table IV, come well outside the limit of experimental error. These, in our opinion, do contain some other fermentable sugar than glucose.

The validity of this conclusion depends entirely on the validity of the methods used, and, therefore, it was only after we felt sure about the methods, particularly the copper method of Folin, that we endeavored to determine whether blood does or does not contain some fermentable sugar other than glucose. All of the determinations were made at least in duplicates; most of them were made in triplicates. In some cases, not reported in Table IV, we also made triplicate determinations before and after the addition of glucose. As we never obtained a difference of over 2 mg. per cent in the triplicate analyses of nine different samples of blood whose sugar content ranged from 78 mg. per cent to 332 mg. per cent, it seems superfluous to give detailed figures for these 54 different determinations.

The sugar values obtained before and after fermentation by the colorimetric ferricyanide method are also reported in Table IV. These values usually serve to confirm the conclusion obtained by comparison of the results from the two copper methods. They also show that it is only by the help of the weakly alkaline copper method of Folin that we could get clear cut evidence of the presence of some unknown fermentable and reducing material.

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# THE EFFECT OF WHOLE SKELETAL MUSCLE ON BLOOD SUGAR IN VITRO.

BY MELVILLE SAHYUN AND CARL L. ALSBERG.

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The question whether or not insulin has to do with the burning of sugar or merely with its transport from the blood into the tissues is not yet satisfactorily answered. A great deal has been done on glycolysis *in vitro* and on the effect of insulin upon it; from the literature one may draw the conclusion that the results were mostly negative.

Glycolysis has been a live topic since 1903 when Cohnheim (1) published his work. Nothing was then known about insulin, though the presence of such a hormone was suspected from the time von Mehring and Minkowski (2) produced diabetes by pancreatectomy. Results similar to those of Cohnheim were obtained by Hirsch (3). Their results were not accepted by the majority of investigators. However in 1907, Hall (4), attacking the problem of glycolysis by using pancreatic extract, glucose, and muscle juice, obtained remarkable results which are now forgotten. Later Levene and Meyer (5) obtained similar results. That careful workers would publish such results without evidence that seemed to them adequate does not appear to us likely. Yet the results that have been reported in the literature ever since insulin was discovered are so overwhelmingly negative that anyone who obtains positive results is prone to question their validity. In consequence, no doubt, workers have hesitated to publish evidence that glycolysis *in vitro* is feasible.

Upon surveying the literature, one observes that (*a*) in most cases, either muscle juice or minced tissues were used, (*b*) to such mixtures were added glucose and often buffers, (*c*) insulin more recently has also been added, whereas in earlier investigations

pancreatic juice or an alcoholic extract of the pancreas was used, and (*d*) the different mixtures were subjected to incubation. There have never been, to our knowledge, any experiments on glycolysis *in vitro* in which whole muscle and hyperglycemic blood of animals treated with epinephrine were used. Such an investigation is reported in the present paper.

We were led to investigate the behavior of muscle tissue and blood from epinephrinized animals because of the great reduction of the glycogen of the skeletal muscle when the animal is treated with epinephrine, as our present work (6), the work of Cori and Cori (7), and of Blatherwick and Sahyun (8) seem to demonstrate very plainly. This suggests that such muscles, being depleted of glycogen, offer more favorable material for the study of glycolysis *in vitro* than those of normal animals. Moreover, after epinephrine injections there is present in the blood a high concentration of sugar, most of which is formed either from the liver, or from the muscle glycogen, or from both. The question, therefore, arises whether or not that part of the sugar mobilized by epinephrine is of the same nature as normal blood sugar. The fact that it disappears in a few hours and leaves the blood with its initial sugar value leads one to think of the possibility that it may be a different form of glucose. The high blood sugar concentration as well as the low glycogen content of the muscles of animals that have received injections of epinephrine, therefore, suggested to us the use of such whole muscle and of blood that contained a high concentration of sugar in studying glycolysis *in vitro*. Insulin was used in concentrated form so that the addition of a few units of U-100 insulin did not introduce any appreciable error into the sugar determinations.

## EXPERIMENTAL.

The following experiments are arranged in three groups. In the first series, the rabbits used were all treated with epinephrine after a 24 hour fast and killed at various intervals of time as indicated in Tables I to III. Since the animals were used for other experiments as well, the results of which will be published later, observations were, therefore, made under various conditions when materials were available. In the experiments of Table I, the muscles of the hind legs were removed, and in so far as cir-

cumstances permitted, whenever the gastrocnemius or the sartorius of the left leg was used for the insulin experiment, that of the right was used as control.   As a large amount of blood was required, it was collected from as many animals as possible (all treated with epinephrine).   Nevertheless, in certain cases, enough was withdrawn from a single animal for the experiments made with its own tissues.   10 to 15 cc. of blood were used for one muscle in most cases.   Unfortunately, in our first series, we ran short of blood, so that the incubation of a similar quantity of blood alone as a control had to be omitted.   The glycogen content of the skeletal muscle was very low, except in those rabbits that were not treated with epinephrine.   The whole muscle, damaged as little as possible, was employed.   In one instance, minced muscle was used, but we think it inadvisable, since mincing damages the muscle fibers.   The tissues were very rapidly removed following the killing of the animal.   In most cases, they kept contracting and relaxing for a short time after being bathed in blood.   Upon the addition of blood, the tissues were thoroughly stirred with a glass rod.   Beakers were used as containers.   In the experiments in which insulin was used, 20 units of Lilly's insulin U-100 (0.2 cc.) were added, followed by stirring.

Combinations of the various tissues were used (Table II) in order to see if there is synergistic action between them, and also to study the effect of various tissues on the blood *in vitro*. If the decrease obtained is uniform, whatever tissues are used, then the decrease observed in the first series (Table I) might be due to microorganisms.

In the third series of experiments, it was the aim to compare the action of normal muscle tissues that were not depleted of their glycogen, with muscle tissues of rabbits depleted of most of their muscle glycogen through epinephrine action.   The blood sugar of animals treated with epinephrine and killed 24 hours after the injections, returns to normal.   In some of these experiments, the sugar content of the blood was raised to an initial value of 200 mg. per 100 cc. by the addition of pure glucose.   In a few experiments of this group, the effect of various tissues upon a solution of glucose in normal saline solution was noted.   Folin and Wu's method of blood sugar determination and Pflüger's method for glycogen were used.

### DISCUSSION.

In Table I, the effect of the addition of insulin is quite noticeable in certain instances and the decrease in glucose cannot possibly be attributed in these experiments to errors of the method, for

TABLE I.

*Effect of Insulin and Tissues of Epinephrinized Rabbits upon Blood Sugar in Vitro.*

| Date. | Experiment No | Muscle glycogen per 100 gm. | Nature of combined materials used | | | Incubation time | Blood sugar per 100 cc | Difference. |
|---|---|---|---|---|---|---|---|---|
| | | | Blood | Muscle. | Insulin. | | | |
| 1929 | | mg. | | | | hrs. | mg | mg. |
| Jan. 21 | 1 | 30 | + | None. | None. | 1 | 310 | |
| | | | + | Minced. | " | 1 | 315 | |
| | | | + | " | " | 4 | 268 | 42 |
| | | | + | Whole. | 20 units. | 1 | 190 | 120 |
| | | | + | " | 20 " | 4 | 143 | 167 |
| Jan. 26 | 2 | 57 | + | None. | None. | 0 | 200 | |
| | | | + | Whole. | " | 2 5 | 178 | 22 |
| | | | + | " | 20 units. | 2.5 | 160 | 40 |
| Jan. 26 | 3 | 55 | + | None. | None. | 0 | 288 | |
| | | | + | Whole. | " | 2 5 | 250 | 38 |
| | | | + | " | 20 units. | 2 5 | 212 | 76 |
| Jan. 29 | 4 | 30 | + | None. | None. | 0 | 250 | |
| | | | + | Whole. | " | 3 | 235 | 25 |
| | | | + | " | 20 units. | 3 | 205 | 45 |
| Jan. 29 | 5 | 27 | + | None. | None. | 0 | 200 | |
| | | | + | Whole. | " | 3 | 185 | 15 |
| | | | + | " | 20 units. | 3 | 121 | 79 |
| Feb. 6 | 6 | 36 | + | None. | None. | 0 | 250 | |
| | | | + | Whole. | " | 3 | 205 | 45 |
| | | | + | " | 20 units. | 3 | 145 | 105 |

determinations were made in duplicate.    Since in all experiments of this series the initial glycogen content of the tissues was very low, insulin might have served either as an accelerator of the utilization of blood sugar by the deglycogenated tissues *in vitro*, or the conditions might have been suitable for insulin to act as

*in vivo.* We have no explanation as yet to offer for its great effect in some experiments and its but slight effect in others, more than to suggest that certain unrecognized conditions are possibly necessary for this peculiar phenomenon. Such conditions might

TABLE II.

*Action of Various Tissues upon Blood Sugar in Vitro.*

| Date. | Experiment No. | Muscle glyco-gen per 100 gm. | Nature of combined materials used. | | | | | | Incubation time. | Blood sugar per 100 cc. | Difference. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Blood. | Muscle. | Heart. | Liver. | Bile. | Insulin. | | | |
| *1929* | | *mg.* | | | | | | | *hrs.* | *mg.* | *mg.* |
| Feb. 5 | 1 | 52 | + | − | − | − | − | None. | 0 | 100 | |
| | 1 a | | + | + | − | − | − | " | 3.5 | 55 | 45 |
| | | | + | + | − | − | − | " | 5 | 35 | 65 |
| | 1 b | | + | + | − | − | + | 20 units. | 3.5 | 83 | 17 |
| | | | + | + | − | − | + | 20    " | 5 | 60 | 40 |
| Feb. 12 | 2 | 30 | + | − | − | − | − | None. | 0 | 100 | |
| | | | + | + | + | − | − | " | 2 | 78 | 22 |
| | | | + | + | + | − | − | " | 3 | 75 | 25 |
| | 2 a | | + | + | + | − | − | " | 2 | 78 | 22 |
| | | | + | + | + | − | − | " | 3 | 79 | 21 |
| | 2 b | | + | + | + | − | − | 20 units. | 2 | 61 | 39 |
| | | | + | + | + | − | − | 20    " | 3 | 61 | 39 |
| | 2 c | | + | + | + | − | − | 20    " | 2 | 80 | 20 |
| | | | + | + | + | − | − | 20    " | 3 | 78 | 22 |
| Feb. 12 | 3 | 31 | + | − | − | − | − | None. | 0 | 105 | |
| | | | + | + | − | − | − | " | 1.5 | 53 | 52 |
| | | | + | + | − | − | − | 20 units. | 1.5 | 55 | 50 |
| | | | + | − | + | − | − | 20    " | 1.5 | 66 | 39 |
| Feb. 12 | 4 | | + | − | − | − | − | None. | 1.5 | 200 | |
| | | | + | − | − | + | − | " | 1.5 | 340 | |
| | | | + | − | − | + | − | " | 3 | 350 | |
| | | | + | − | − | + | − | 20 units. | 1.5 | 340 | |
| | | | + | − | − | + | − | 20    " | 3 | 358 | |

be worth studying; but we are not now ready to generalize in regard to the data of Table I.

When various tissues were employed instead of whole skeletal muscle, or in combination with whole skeletal muscle, results were

TABLE III.

*Action of Deglycogenated and Normal Muscle upon Blood Sugar in Vitro.*

| Date. | Experiment No. | Muscle glycogen per 100 gm. | Nature of combined materials used. | | | | | | Incubation time. | Blood sugar per 100 cc. | Difference. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Blood. | Glucose. | Muscle. | Heart. | Saline and glucose. | Insulin. | | | |
| *1929* | | mg. | | | | | | | hrs. | mg. | mg. |
| Feb. 16 | 1* | | + | + | − | − | − | None. | 0 | 200 | |
| | | | + | + | − | − | − | " | 2 | 185 | |
| | | | + | + | − | − | − | " | 3 | 135 | 65 |
| | 1 a* | 25 | + | + | − | + | − | 20 units. | 2 | 160 | |
| | | | + | + | − | + | − | 20  " | 3 | 140 | 60 |
| | 1 b* | | + | + | − | + | − | None. | 2 | 200 | |
| | | | + | + | − | + | − | " | 3 | 140 | 60 |
| | 1 c* | | + | + | + | − | − | " | 2 | 129 | |
| | | | + | + | + | − | − | " | 3 | 87 | 113 |
| | 1 d* | | + | + | + | − | − | " | 2 | 143 | |
| | | | + | + | + | − | − | " | 3 | 93 | 107 |
| | 1 e* | | + | + | + | − | − | 20 units. | 2 | 128 | |
| | | | + | + | + | − | − | 20   " | 3 | 77 | 123 |
| | 1 f* | | + | + | + | − | − | 20   " | 2 | 133 | |
| | | | + | + | + | − | − | 20   " | 3 | 91 | 109 |
| Feb. 23 | 2† | | + | + | − | − | − | None. | 0 | 200 | |
| | | | + | + | − | − | − | " | 2 | 165 | |
| | | | + | + | − | − | − | " | 3 | 143 | 67 |
| | 2 a† | 152 | + | + | − | + | − | 20 units. | 2 | 170 | |
| | | | + | + | − | + | − | 20   " | 3 | 170 | 30 |
| | 2 b† | | + | + | − | + | − | 20   " | 2 | 173 | |
| | | | + | + | − | + | − | 20   " | 3 | 175 | 25 |
| | 2 c† | | + | + | − | + | − | None. | 2 | 180 | |
| | | | + | + | − | + | − | " | 3 | 155 | 45 |
| | 2 d† | 135 | + | + | + | − | − | " | 2 | 167 | |
| | | | + | + | + | − | − | " | 3 | 143 | 67 |
| | 2 e† | | + | + | + | − | − | 20 units. | 2 | 167 | |
| | | | + | + | + | − | − | 20   " | 3 | 143 | 67 |
| | 2 f† | | + | + | + | − | − | 20   " | 2 | 173 | |
| | | | + | + | + | − | − | 20   " | 3 | 175 | 25 |
| Feb. 23 | 3 | | − | − | − | − | + | None. | 0 | 200 | |
| | | | − | − | − | − | + | " | 2 | 200 | |
| | | | − | − | − | − | + | " | 3 | 200 | |
| | 3 a | | − | − | − | + | + | " | 2 | 200 | |
| | | | − | − | − | + | + | " | 3 | 170 | 30 |

TABLE III—*Concluded.*

| Date. | Experiment No. | Muscle glyco- gen per 100 gm. | Nature of combined materials used. | | | | | | Incubation time. | Blood sugar per 100 cc. | Difference. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Blood. | Glucose. | Muscle. | Heart. | Saline and glucose. | Insulin. | | | |
| *1929* | | *mg.* | | | | | | | *hrs.* | *mg.* | *mg.* |
| Feb. 23 | 3 b‡ | 245 | — | — | — | + | + | 20 units. | 2 | 200 | |
| | | | — | — | — | + | + | 20   " | 3 | 170 | 30 |
| | 3 c‡ | | — | — | + | — | + | None. | 2 | 212 | |
| | | | — | — | + | — | + | " | 3 | 180 | 20 |
| | 3 d‡ | | — | — | + | — | + | 20 units. | 2 | 215 | |
| | | | — | — | + | — | + | 20   " | 3 | 180 | 20 |
| | 3 e* | 16 | — | — | + | — | + | None. | 2 | 182 | |
| | | | — | — | + | — | + | " | 3 | 148 | 62 |
| | 3 f* | | — | — | + | — | + | 20 units. | 2 | 174 | |
| | | | — | — | + | — | + | 20   " | 3 | 148 | 62 |

\* Animals received epinephrine.

† Control animals that did not receive epinephrine fasted 48 hours.

‡ Control; no epinephrine.

variable (Table II). The liver, for example, caused an increase in the quantity of sugar after incubation, as has previously been reported by others (9). The heart, as well as the kidneys, had little or no effect on the blood sugar *in vitro*. The decrease recorded, as is obvious from results reported in Table III, is similar to that observed in the incubation of the blood alone. In the single experiment made with bile, the effect of this fluid is noticeable. Not only has insulin action, or rather that of the deglycogenated tissues, been inhibited, but reduction of blood sugar has also been quite retarded.

The conclusion at which we arrive becomes clear in the last series of experiments in which a comparison was made between deglycogenated whole skeletal muscle and normal glycogen-containing muscle. When blood alone was incubated, the blood sugar dropped from an initial content of 0.200 per cent to 0.135 and 0.143 per cent, respectively, whether or not the blood was derived from epinephrinized or normal animals. This decrease is quite possibly due to the utilization of sugar by the red corpuscles of the blood and almost certainly not to the action of micro-organisms. This becomes obvious when in the same quantity of

blood a whole heart was suspended and incubated. The final decrease reached approximately the same level (in one instance 0.140 per cent and in another 0.170 per cent), as can be seen in Table III. But when the experiment was performed with deglycogenated skeletal muscle, the drop in blood sugar at the end of the 3rd hour reached 0.087 per cent and 0.093 per cent, whether insulin had been added or not. Under such circumstances insulin had no effect. On the other hand, when normal skeletal muscle was used, the decrease at the end of the 3rd hour was as small as when blood alone was incubated for a similar period of time. This striking phenomenon also took place when a mixture of saline solution and glucose was used instead of blood. The incubation of such a mixture by itself did not decrease the quantity of glucose. Consequently, the conclusion might be drawn that deglycogenated whole skeletal muscle has the power of utilizing glucose *in vitro*. Whether the glucose that disappeared was burned or changed to glycogen, we are not yet in a position to state. If, indeed, one of the factors that determines the removal of glucose from blood by muscle is the condition of the muscle, of which its glycogen content is an index, then, perhaps, the reason why neither heart nor kidney removes glycogen is to be attributed to the possibility that epinephrine does not deglycogenate them. This is quite probable, at least for the heart, since it is well known that even in advanced fasting the heart does not suffer much loss of weight as compared with other organs. These, however, are questions now under investigation. We hope, also, through further studies, to throw light on the behavior of deglycogenated muscle and glucose *in vitro*.

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# THE OPTICAL PROPERTIES OF SOME AMINO ACIDS.

## II. ARGININE AND HISTIDINE.

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In a former paper (1) the writer discussed the optical properties of eleven of the crystalline amino acids. Recently (2, 3), Vickery and Leavenworth have succeeded in obtaining three additional acids, namely arginine, histidine, and lysine, in the crystalline form, two of which—arginine and histidine—appeared to be suitable for study by the optical immersion method. The object of this paper is to supplement data already obtained (1).

The author desires to acknowledge the assistance of Dr. Hubert B. Vickery, of the Connecticut Agricultural Experiment Station, who was kind enough to furnish the crystalline acids for this study.

## Arginine.

### Crystallized from Water, Dihydrate.

*In Ordinary Light.*—For study by the optical immersion method, this material is broken up into fragments of irregular shape.

*In Parallel Polarized Light, Crossed Nicols.*—Double refraction is very strong. Like the anhydrous form, some plates show brilliant colors of the second order, while others are so thin that only first order white is shown.

*In Convergent Polarized Light, Crossed Nicols.*—Biaxial interference figures are rather common, particularly plates perpendicular to an optic axis.

*Refractive Indices.*—$n_\alpha$ = 1.528; $n_\beta$ = 1.549; $n_\gamma$ = 1.579; $n_\gamma - n_\alpha$ = 0.051. All ±0.003.

*Diagnostic Characters.*—The frequency with which plates show one optic axis up in the interference figure, also the minimum and maximum refractive index values ($n_\alpha$ = 1.528, $n_\gamma$ = 1.579) are useful for determinative purposes and serve to differentiate this acid from the other amino acids.

*Crystallized from 66 Per Cent Alcohol.*

*In Ordinary Light.*—Thin, colorless plates; outline various.

*In Parallel Polarized Light, Crossed Nicols.*—Double refraction is very strong, some plates showing brilliant colors of the second order, although many of the plates are so thin that only first order white and yellow are shown.

*In Convergent Polarized Light, Crossed Nicols.*—Biaxial, but no interference figures are visible on most of the plates.

*Refractive Indices.*—$n_\alpha = 1.548$; $n_\beta = 1.562$; $n_\gamma = 1.610$; $n_\gamma - n_\alpha = 0.062$; $n_\beta$ usually shown on plates on edge.   All $\pm 0.003$.

*Diagnostic Characters.*—The thin, platy character, also the frequency with which all three significant indices of refraction can be located are useful for determinative purposes.

### *Histidine.*

### *Crystallized from Water.*

*In Ordinary Light.*—Colorless plates usually with irregular outline; occasionally elongated fragments.

*In Parallel Polarized Light, Crossed Nicols.*—Double refraction is extremely strong, the thicker plates showing brilliant colors of second order, the thinner ones first order white.   Sign of elongation on the few fragments with definite elongation—; extinction, straight.

*In Convergent Polarized Light, Crossed Nicols.*—Biaxial, but portions of interference figures rarely visible.

*Refractive Indices.*—$n_\alpha = 1.520$; $n_\beta = $ indeterminable; $n_\gamma = 1.610$; $n_\gamma - n_\alpha = 0.090$; both $\pm 0.003$.

*Diagnostic Characters.*—The values $n_\alpha$ (1.520) and $n_\gamma$ (1.610) are most significant for diagnostic purposes.

#### SUMMARY.

Optical data on crystalline arginine and histidine have been presented to supplement data already published on other crystalline amino acids.

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# ON THE MECHANISM OF PHLORHIZIN DIABETES.

## III. THE EFFECT OF PHLORHIZIN UPON GLYCOGEN STORAGE BY DOGS WITH LIGATED URETERS.

By THOMAS P. NASH, Jr.

*(From the Department of Chemistry, University of Tennessee College of Medicine, Memphis.)*

Among the more recent contributions to the problem of the mechanism of phlorhizin diabetes is the publication by Deuel, Wilson, and Milhorat (1) in which they support the hypothesis that the action of phlorhizin is exclusively renal. These authors confirm and extend the earlier observation of Wierzuchowski (2) that a rise in respiratory quotient follows administration of sugar to the phlorhizinized dog. They report, also, data of various experiments upon nephrectomized dogs which are in general agreement with the view that phlorhizin does not effect any direct or intrinsic impairment of the normal carbohydrate process in animal tissues. Administration of phlorhizin to dogs whose kidneys had been extirpated did not reduce the fasting R.Q. to the diabetic level or prevent a substantial rise in R.Q. after glucose feeding; in such animals the blood sugar remained practically at the normal level, and there was no detectable influence of phlorhizin upon the rate of increase of non-protein nitrogen in the blood.

Such results are difficult to reconcile with any direct extrarenal mechanism of phlorhizin action. It is now possible, as Deuel, Wilson, and Milhorat suggest, to explain as secondary results of the increased kidney permeability to glucose some of the behaviors of the phlorhizinized animal which hitherto have been interpreted as indicating a direct intervention of phlorhizin in the carbohydrate metabolism. Thus, the ketosis and acidosis which characterize phlorhizin diabetes may result from the lowered concentration of carbohydrate in the tissues, and, in turn, may

interfere with the glycogenic function when carbohydrate is administered.   Again, studies of insulin mechanism have indicated a relationship between blood sugar concentration and insulin production, and it has been observed that fasting an animal lowers its carbohydrate tolerance.   Either or both of these factors may be invoked to account for the essential failure of the phlorhizinized animal which retains its renal function to oxidize glucose, as well as the more pronounced and prolonged hyperglycemia[1]—as contrasted with the response in a normal animal—which follows glucose feeding.

On the other hand, a considerable and important weight of evidence[2] against the view of an exclusively renal site of phlorhizin action cannot be disposed of so summarily.   It is the purpose of the present paper not to argue the issue, however, but to report experiments carried out in an effort to determine whether phlorhizin has *no effect whatsoever* upon an animal whose renal function has been abolished.

It does not appear to the present author that the results obtained by Deuel, Wilson, and Milhorat dispose of such a possibility.   In their report may be noted, for example:

1. Despite their main thesis, the authors suggest an effect of phlorhizin upon protein metabolism which is not related to a fall in the blood sugar and hence is inferred to be a direct, extra-renal action.   An increased rate of protein metabolism is observed to begin while the blood sugar value is still normal, *but is just on the point of beginning to fall* some 12 hours after the administration of phlorhizin; and as the blood sugar begins to fall the protein metabolism increases rapidly.   Here, it appears to the present author, the more likely explanation is that an increase in protein

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[1] This phenomenon has been confirmed repeatedly (*cf.* for example, Nash and Benedict (3), Kempner (4), Deuel, Wilson, and Milhorat (1)). It is especially remarkable since it coincides with practically quantitative elimination of the fed sugar.   We have carried out unreported experiments in which equal amounts of glucose were administered to the same dog after corresponding periods of simple fasting and fasting with phlorhizin, and have thus demonstrated again that in the phlorhizinized state the hyperglycemia reaches higher values and endures 2 to 3 hours longer.

[2] *Cf.* for example, Nash (5), Kempner (4), Rapport and Ralli (6), Beck (7), Kastler (8), Galambos (9), Peskin (10).

metabolism coincides with and is due to a depletion of the glycogen reserves of the tissues.

2. When glucose was ingested by phlorhizinized dogs which retained their kidneys, the sum of the extra sugar excreted in the urine and that indicated by the respiratory exchange to have been oxidized exceeded by from 10 to 20 per cent the amount introduced. The authors point out that this discrepancy exceeds the experimental error in the methods employed. At least, such a discrepancy is a further caution to reserve in accepting at face value respiratory measurements obtained under grossly abnormal and complex conditions.

3. The nephrectomized dogs employed received phlorhizin only *after* operation. Apparently, also, the animals were not fasted prior to nephrectomy. Because of the generally very poor absorption in such subjects it may be questioned whether phlorhizin introduced subcutaneously was rapidly or completely absorbed. It is likewise questionable whether appreciable carbohydrate stores in the tissues might not protect the metabolic process against phlorhizin intervention, which, if once accomplished, might equally resist displacement by carbohydrate.

4. The failure of sugar to accumulate in the blood of the nephrectomized and phlorhizinized animal is not, *per se*, evidence that phlorhizin has not interfered with the oxidation of carbohydrate. On the contrary, it might be expected[3] that when the blood sugar reaches a normal level the glycogen-storing mechanism would come into operation. The authors recognize this possibility, but discredit it on the basis of a calculation which is not supported by their data. The calculation (Dog 178) assumes a rate of protein breakdown in the nephrectomized animal equivalent to that in the phlorhizinized animal with intact kidneys, and thus arrives at the value of 294 gm. of glycogen as the amount which should have been present in the whole animal at time of death if none had been oxidized. Actually, 22 gm. of glycogen were found. Inspection of the rise in the non-protein nitrogen of the blood of this animal during the 4 days of the experiment discloses an increase from 46 mg. to 280 mg. per 100 cc., or a net increase of 0.234 per cent. Even if it be assumed that all the tissues of the animal (weight 19.2 kilos)

[3] *Cf.* Nash (11).

would show the same increase as the blood, we can calculate a total nitrogen metabolism for the period of only 45 gm., equivalent to 281 gm. of protein, or 163 gm. of glucose. A much more reasonable calculation could be based upon the probable nitrogen excretion of a normal fasting dog of the same weight. This would be approximately 3 gm. of nitrogen per day.[4] On this basis, and without consideration of the glycogen present in the tissues at the start of the experiment, 43 gm. of sugar might have come from the protein metabolism of the period. This is much nearer the amount of glycogen present in the one dog analyzed, and compares with 6.86 gm. of glycogen in the single control dog which was nephrectomized but did not receive phlorhizin.

5. Failure to find a much more rapid rise in the non-protein nitrogen of the nephrectomized animal after administration of phlorhizin likewise is not valid evidence of phlorhizin inactivity. In setting up as a test of phlorhizin effect upon the nephrectomized animal a progressive increase in protein metabolism of the order found in the phlorhizinized animal with functioning kidneys, the authors ignore the largest single factor concerned, namely Ringer's (12) "dextrose nitrogen." It is precisely this factor which would be abolished by a return of the blood sugar to normal levels.

In consideration of the possibilities suggested in the foregoing, and particularly because of the apparent increase in glycogen found by Deuel, Wilson, and Milhorat when phlorhizin was administered to a nephrectomized dog, it appeared desirable to extend the study of the glycogenic action of phlorhizin.[5] While such a study has failed to demonstrate conclusively any influence of phlorhizin upon glycogen storage, the experiments appear worthy of recording not only because of this finding, but also because they contradict a very general impression that fasting combined with exposure, exercise, adrenalin administration, and similar procedures

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[4] In our experiments, for example, for the 4th day of fasting Dog 34, weight 12 kilos, excreted 3 47 gm. of nitrogen in the urine; Dog 35, weight 11.5 kilos, excreted 2 94 gm. of nitrogen.

[5] Epstein and Baehr (13), working with nephrectomized, phlorhizinized cats, have claimed that phlorhizin stimulates the accumulation of glycogen in the liver.

constitute an effective deglycogenizing routine, to be relied upon for establishing a control state.[6]

## Methods and General Procedure.

Unless otherwise specified, the analytical methods and experimental procedures were those employed previously in the study of glycogen formation in phlorhizinized dogs (11). Blood sugar was determined by the Benedict method (19), and frequently, also, by the methods of Folin and Wu (20), Benedict (21), and Harned (22). With few exceptions blood was taken from the jugular vein. Sugar of muscle and liver was determined in the manner described by Harned (22).

Bilateral ureteral ligation was employed rather than kidney extirpation in order to test the possibilities that the blood sugar is altered in quality during its passage through the phlorhizinized kidney, and that the kidney is so affected by phlorhizin as to contribute to the circulation some product which interferes with sugar combustion. A further advantage of this procedure was the opportunity to determine whether phlorhizin had been absorbed in amount sufficient to affect at least the renal tissue; without exception at autopsy in those animals which received phlorhizin at any stage of the experiment, fluid aspirated from the ureter between the point of ligation and the kidney reduced Benedict's qualitative sugar reagent. The operation was performed under ether anesthesia through a median abdominal incision, the success and sterility of the operation being checked at autopsy. All of the animals of the series were fasted both before and after

[6] Geiger and Schmidt (14) found in dogs fasted and phlorhizinized for 4 days values of liver glycogen from 0.004 to 0.016 per cent, while the muscle glycogen ranged from 0.197 to 0.320 per cent. When adrenalin also was administered, no further depletion of liver glycogen occurred, but the muscle glycogen fell to very low levels, varying between 0.004 and 0.013 per cent. Bodo and Marks (15) also have recently reported exceedingly low initial values for the liver glycogen of dogs fasted for 48 hours. On the other hand, Nitzescu and Benetato (16) find that dogs kept 39 to 40 days without food still contained traces of glycogen in the muscle, and from 0.240 to 0.875 per cent of glycogen in the liver, with an average for four dogs of 0.64 per cent. Wertheimer (17) states that a liver can never be found to be completely free of glycogen. Markowitz, Mann, and Bollman (18), likewise, call attention to the difficulty of deglycogenizing an animal.

TABLE I.

*Glycogen Formation in Fasting, Ureter-Ligated Dogs, with and without Phlorhizin.*

| Dog No | Time after last feeding | Time after operation | Weight before operation | Weight at death | Weight of liver | Blood non-protein N per 100 cc | | Glycogen | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Before operation | At death | Muscle | Liver |

**Group 1-a   No phlorhizin given before or after operation.   Complete fasting.**

| | *days* | *hrs* | *kg* | *kg* | *gm* | *mg* | *mg* | *per cent* | *per cent* |
|---|---|---|---|---|---|---|---|---|---|
| 31 | 7 | 72 | | 11 5 | 340 | 39 | 244 | 0 276 | 0 308 |
| 32* | 7 | 78 | 10 2 | | 240 | 43 | 35 | 0 385 | 0 879 |
| 44 | 10 | 73 | 11 7 | 11 4 | 345 | 46 | 206 | 0 481 | 2 23 |

**Group 1-b   No phlorhizin given before or after operation.   Complete fasting except for glucose given after operation**

| 35 | 7 | 78 | 11 6 | 11 1 | 327 | 40 | 167 | 0 508 | 2 63 |
|---|---|---|---|---|---|---|---|---|---|
| 47† | 10 | 76 | 12 7 | 12 1 | 328 | 29 | 179 | 0 252 | 0 370 |
| 49† | 10 | 76 | 12 3 | 12 2 | 359 | 45 | 205 | 0 395 | 2 22 |

**Group 2-a   Phlorhizin given only after operation   Complete fasting**

| 29 | 7 | 78 | 12 0 | 11 3 | 340 | 30 | 250 | 0 246 | 1 20 |
|---|---|---|---|---|---|---|---|---|---|
| 30 | 6 | 78 | 16 0 | 15 2 | 513 | 24 | 196 | 0 382 | 1 98 |
| 45† | 9 | 74 | 14 3 | 13 8 | 344 | 57 | 170 | 0 351 | 1 14 |

**Group 2-b.   Phlorhizin given only after operation.   Complete fasting except for glucose given after operation**

| 34 | 7 | 75 | 12 3 | | 317 | 34 | 183 | 0 572 | 2 52 |
|---|---|---|---|---|---|---|---|---|---|

**Group 3-a.   Phlorhizin given before and after operation.   Complete fasting.**

| 36 | 11 | 120 | 17 3 | 15 6 | 447 | 46 | 273 | 0 544 | 1.14 |
|---|---|---|---|---|---|---|---|---|---|
| 40 | 5 | 30 | 23 5 | | 467 | 39 | 96 | 0 583 | 2 01 |
| 42 | 9 | 75 | 11 0 | 10 7 | 378 | 44 | 257 | 0 328 | 4 05 |
| 43 | 10 | 73 | 13 5 | 12 5 | 445 | 58 | 290 | 0 414 | 3 10 |
| 46† | 9 | 54 | 12 3 | 11 1 | 409 | 42 | 220 | 0 183 | 3 24 |

**Group 3-b.   Phlorhizin given before and after operation.   Complete fasting except for glucose given after operation**

| 41 | 8 | 77 | 15 6 | | 434 | 44 | 218 | 0 484 | 5.71 |
|---|---|---|---|---|---|---|---|---|---|
| 48† | 11 | 75 | 15 1 | 14 5 | 430 | 45 | 250 | 0 210 | 2 88 |
| 50† | 10 | 75 | | 8 5 | 316 | 55 | 290 | 0 267 | 3 69 |

\* Ureter ligated on one side only.

† Special procedures calculated to deglycogenize animal prior to operation, as detailed in protocols.

TABLE I—*Concluded.*

Preoperative data of dogs phlorhizinized before operation are shown in Table II. Preoperative procedures in certain other cases, and postoperative details of phlorhizin and glucose administration, were as follows:

*Dogs 29 and 30.*—1 gm. of phlorhizin in olive oil given subcutaneously, 6, 22, and 70 hours after operation. 1 gm. of phlorhizin in 1 per cent sodium carbonate given subcutaneously, 6 and 46 hours after operation.

*Dog 34.*—Phlorhizin given as with Dogs 29 and 30. 10 gm. of glucose subcutaneously, 44 hours after operation. 10 gm. of glucose intravenously, 68 hours after operation.

*Dog 35.*—Glucose given as with Dog 34.

*Dogs 36, 40, 42, and 43.*—1 gm. of phlorhizin in olive oil subcutaneously, daily after operation.

*Dog 41.*—Phlorhizin given as with Dog 36. 10 gm. of glucose intravenously, 48 hours after operation.

*Dog 45.*—Ice water bath followed by exposure to cold for 4 hours on day preceding operation. 0.2 gm. of phlorhizin in 10 cc. of warm water intravenously, 6, 22, 46, and 70 hours after operation.

*Dog 46.*—0.2 gm. of phlorhizin in 10 cc. of warm water intravenously, 22 and 46 hours after operation.

*Dog 47.*—On 3rd day preceding operation, two subcutaneous injections, 1 hour apart, of 1.0 cc. of 1:1000 adrenalin, followed by ice bath and exposure to cold for 2 hours. On 2nd day preceding operation, 0.75 cc. of 1:1000 adrenalin, subcutaneously. 10 gm. of glucose intravenously, 22 and 46 hours after operation.

*Dog 48.*—10 gm. of glucose with 0.2 gm. of phlorhizin intravenously, 22 and 46 hours after operation. 0.2 gm. of phlorhizin intravenously, 70 hours after operation.

*Dog 49.*—Preoperative procedure as with Dog 47. 10 gm. of glucose subcutaneously, 24 hours after operation. 15 gm. of glucose subcutaneously, and 10 gm. of glucose intravenously, 48 hours after operation.

*Dog 50.*—10 gm. of glucose with 0.2 gm. of phlorhizin intravenously, 22 hours after operation. 5 gm. of glucose with 0.2 gm. of phlorhizin intravenously, and 10 gm. of glucose subcutaneously, 46 hours after operation. 10 gm. of glucose intravenously, 52 hours after operation. 0.2 gm. of phlorhizin intravenously, 72 hours after operation.

operation except for the administration of glucose in some cases. Glucose when given was by jugular vein (occasionally, also, subcutaneously) in warm aqueous 50 per cent solution.[7] Phlorhizin was given subcutaneously in olive oil or 1 per cent sodium carbonate solution; after operation, phlorhizin was also given intravenously, dissolved in warm water or glucose solution.

[7] Vomiting was an almost invariable and immediate response to intravenous injection of 20 cc. of aqueous 50 per cent glucose solution.

TABLE II.

*Urine Analyses for Dogs Phlorhizinized before Operation. Daily Subcutaneous Injections of 1 Gm. of Phlorhizin in Olive Oil. Fasting.*

| Days after phlorhizin. | Length of period. | "Total acetone." | Glucose | Nitrogen. | D:N ratio. | Remarks. |
|---|---|---|---|---|---|---|
| | *hrs* | *gm.* | *gm.* | *gm.* | | |

Dog 36; weight, 17.8 kilos; preliminary fast, 3 days.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 24 | 0.170 | 33.68 | 6 47 | 5.20 | Very fat. |
| 2 | 24 | 2 164 | 32.44 | 8 71 | 3.72 | |
| 3 | 24 | 3.576 | 32 00 | 9.67 | 3 31 | |

Dog 40; weight, 23.7 kilos; preliminary fast, 36 hrs.

| | | | | | | |
|---|---|---|---|---|---|---|
| 2 | 24 | 0.198 | 41 34 | 10 55 | 3 92 | |
| 3 | 24 | 0.726 | 40 38 | 11.86 | 3.40 | |

Dog 41; weight, 16.8 kilos; preliminary fast, 2 days.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 24 | | 34.52 | 7.80 | 4.42 | |
| 2 | 24 | | 34.60 | 10 82 | 3.19 | |
| 3 | 24 | 0 560 | 34.84 | 11.18 | 3.12 | |

Dog 42; weight, 11.9 kilos; preliminary fast, 3 days.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 24 | 0.282 | 32.68 | 7.57 | 4.31 | |
| 2 | 24 | 0.770 | 39.04 | 11.75 | 3.32 | |
| 3 | 24 | 1 502 | 37.36 | 11.44 | 3.26 | |

Dog 43; weight, 14.5 kilos; preliminary fast, 4 days.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 24 | 0.130 | 37.24 | 7.43 | 5.01 | |
| 2 | 24 | 1 124 | 37.32 | 10 34 | 3.61 | |
| 3 | 24 | 2 93 | 40 49 | 11.83 | 3 42 | |

Dog 46; weight, 13.8 kilos; preliminary fast, 4 days. 20 hrs. before first phlorhizin injection dog was given a bath in ice water and placed, wet, in a large refrigerator for 2 hrs. at 5–6°.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 22 | 3.37 | 29.22 | 7.25 | 4.03 | |
| 1 | 2 | 0.289 | 2.80 | 0.835 | 3.35 | 0.5 cc. 1:1000 adrenalin, subcutaneously. |
| 2 | 4 | 0.669 | 5.71 | 1.62 | 3.52 | "          " |
| 2 | 6 | 0.707 | 13.23 | 2.41 | 5.49 | |
| 2 | 14 | 3 34 | 16.32 | 5.08 | 3.21 | |
| 3 | 24 | 2.95 | 26.00 | 8.26 | 3.15 | Dog very weak. |

TABLE II—*Concluded.*

| Days after phlorhizin. | Length of period. | "Total acetone." | Glucose. | Nitrogen. | D:N ratio. | Remarks. |
|---|---|---|---|---|---|---|

Dog 48; weight, 16.5 kilos; preliminary fast, 5 days.  18 hrs. before first phlorhizin injection dog was placed, wet, in a large refrigerator for 2 hrs. at 5–6°.

| | *hrs.* | *gm.* | *gm.* | *gm.* | | |
|---|---|---|---|---|---|---|
| 1 | 24 | 0.232 | 39.84 | 5.80 | 6.88 | 1.0 cc. 1:1000 adrenalin, subcutaneously. |
| 2 | 4 | 0 212 | 9.54 | 1.17 | 8.16 | "          " |
| 2 | 6 | 0.092 | 22.98 | 1.75 | 13.15 | |
| 2 | 14 | 1.66 | 25.04 | 5.52 | 4.55 | |
| 3 | 24 | 4.48 | 39.70 | 12.52 | 3.17 | Dog in splendid condition. |

Dog 50; weight, 11.3 kilos; preliminary fast, 4 days.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 4 | | 6.80 | 1.12 | 6 07 | 1.0 cc. 1:1000 adrenalin, subcutaneously. |
| 1 | 20 | 1 19 | 44.76 | 7.22 | 6 20 | "          " |
| 2 | 4 | 0 445 | 8.48 | 2 00 | 4.24 | "          " |
| 2 | 7 | 1 25 | 10.54 | 3.34 | 3.16 | Rapid breathing; animal inert. |
| 2 | 13 | 1.12 | 15.79 | 6.57 | 2.40 | |
| 3 | 24 | 0.736 | 31.36 | 11.33 | 2.77 | Dog in good condition. |

Usually the experiment was terminated while the dog was still reasonably active and apparently in condition to survive for at least another day.   The dog was killed by a blow on the head, followed by a chloroform mask.   Tissues were taken as rapidly as possible by three operators.   100 gm. portions were employed for glycogen estimations, and 50 gm. portions for sugar.   Selection and dissection of definite leg muscles was sacrificed to speed of removal; but with the large portions taken, from both sides, and always from the same approximate location, it is believed that any error due to unequal distribution of glycogen (18) is substantially compensated. After striking the animal, the time required to remove, weigh, and introduce the liver samples into the hot extractives varied between 1 and 2 minutes; for the muscle the time was from 2 to 4 minutes.

### EXPERIMENTAL.

Animals which developed postoperative infections, or which for any other reason are not considered satisfactory subjects, were

discarded; eighteen dogs comprise the series reported in the present paper. These may be classified in three groups: (1) Controls, receiving no phlorhizin either before or after operation. (2) Those receiving phlorhizin after operation only. (3) Those receiving phlorhizin both before and after operation. Each group



CHART 1. The blood sugar of dogs, with and without phlorhizin, after ligation of the ureters.

is again separable into animals which received glucose after operation, and animals whose fast was complete. Some animals of each group were treated in various ways, as detailed in Tables I and II, in an effort to reduce the glycogen reserves of the tissues to minimal values before operation. Glucose was administered in some cases in an effort to magnify any differences that might

exist between the capacities of phlorhizinized and non-phlor-
hizinized animals to dispose of carbohydrate which, otherwise,
was restricted to that originating from the protein metabolism.

*1. Effect of Phlorhizin upon Blood Sugar Level of Dogs with
Ligated Ureters, during Fasting and after Glucose.*

No effect of phlorhizin upon the blood sugar level of fasting,
anuric dogs has been observed as also found previously by Deuel,
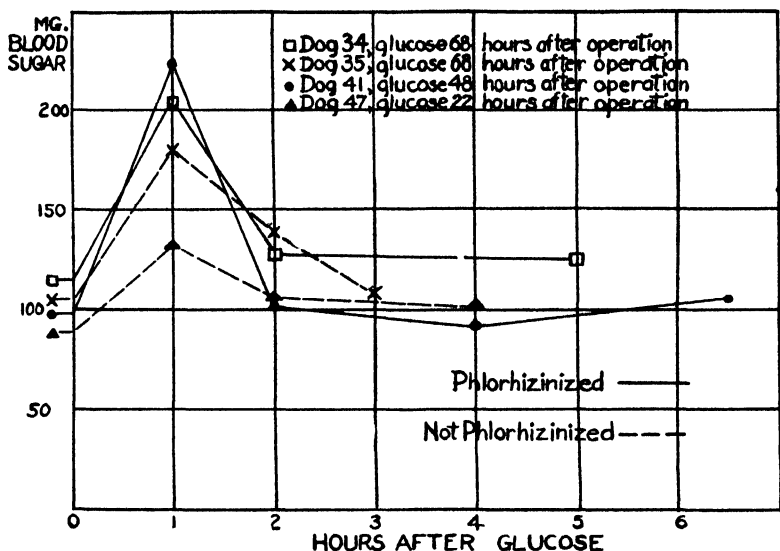


CHART 2. The blood sugar after intravenous injection of 10 gm. of
glucose in phlorhizinized and non-phlorhizinized dogs with ligated ureters.

Wilson, and Milhorat. In Chart 1 are plotted the blood sugar
values of several experiments at various intervals after tying off
the ureters. Dogs 31 and 44 did not receive phlorhizin at any
stage of the experiment, whereas Dogs 36 and 42 were completely
phlorhizinized before operation and continued to receive phlorhizin
thereafter. The rise in blood sugar which follows anesthesia was
generally somewhat higher and more prolonged in the phlorhizinized
animals, but the results given may be taken as entirely typical of
the whole series in failing to show any sustained or progressive

increases in blood sugar concentration.  As already suggested, however, such increases need not be anticipated as a necessary consequence of failure in carbohydrate oxidation.

When equivalent amounts of glucose are given intravenously the degree and duration of the subsequent hyperglycemia are not significantly more pronounced in dogs which also have received phlorhizin.  Several typical comparisons are shown in Chart 2. These findings contrast with those obtained in dogs which retain kidney function.[1]

### 2. Effect of Phlorhizin upon Non-Protein Nitrogen of Blood of Dogs with Ligated Ureters.

Values of the blood non-protein nitrogen just before operation and at time of death are given in Table I.  In every experiment numerous intermediate observations were made, but the intermediate values are not charted since, within reasonable limits of error, when plotted against time they fall upon the straight line drawn through the initial and final values.  In none of the experiments was observed, as a result of phlorhizin administration, a more rapid rate of increase in the non-protein nitrogen of the blood after ureteral ligation.  This is true even for dogs phlorhizinized during several days prior to operation, and having at the time of operation a nitrogen metabolism 3 to 4 times larger than the normal fasting value.  Thus, for example, Dog 31, weighing 11.5 kilos, receiving no phlorhizin before or after operation, and killed 72 hours after operation, had an increase in blood non-protein nitrogen from 39 to 244 mg. per 100 cc.  Dog 42, weighing 10.7 kilos, phlorhizinized before and after operation, and killed 75 hours after operation, showed an increase in non-protein nitrogen from 44 to 257 mg. per 100 cc. of blood.  During the 24 hours preceding operation (the 3rd day of the phlorhizin period), Dog 42 excreted in the urine 11.44 gm. of nitrogen (Table II).  At operation, therefore, the nitrogen breakdown in Dog 42 was at the rate of at least 475 mg. per hour.  Had this rate of nitrogen metabolism continued after operation, and had the nitrogen accumulation been confined to the blood, it may be calculated roughly that the blood non-protein nitrogen would have doubled in the 1st hour. Actually, 6 hours after operation, the blood non-protein nitrogen

was 62 mg. per 100 cc., a value which represents the same rate of accumulation as obtained in subsequent periods.

The *immediate* reversion of the nitrogen metabolism to a normal fasting level when the hypoglycemia of the phlorhizinized animal is abolished is somewhat surprising even if it be granted that phlorhizin is not involved in any generalized tissue process. Certainly this fact is not in harmony with the view of Deuel, Wilson, and Milhorat that phlorhizin directly stimulates protein metabolism.

Another somewhat surprising result in these experiments is the relatively insignificant effect of glucose administration upon the rate of non-protein nitrogen accumulation in the blood of anuric dogs regardless of whether phlorhizin is also given. Immediately after intravenous injection of 20 cc. of 50 per cent glucose solution there is apparent some consistent *small* disturbance in the non-protein nitrogen values, but later values again fall upon an extrapolation of the earlier values.

In this connection it may be remarked again that no such considerable differences as postulated by Deuel, Wilson, and Milhorat should be anticipated between the rates of accumulation of non-protein nitrogen in the blood of fasting anuric non-phlorhizinized and phlorhizinized dogs, even if carbohydrate oxidation is completely abolished in the latter. In the latter circumstance, the only extra demand upon the protein metabolism would be for energy purposes because of failure to derive energy from the sugar arising from protein. It would seem improbable that the entire energy deficit thus created would be made up at the expense of protein; some share of the extra burden would be borne by fat. Furthermore, of the total fasting nitrogen metabolism, a certain substantial fraction constitutes the inevitable "wear and tear" quota, which is, presumably, independent of dynamogenetic considerations. In the normal fasting dog, protein probably does not contribute more than 15 to 20 per cent of the total calorific requirement. If 58 per cent of the protein calories should become unavailable by virtue of phlorhizin intervention the degree to which protein catabolism would thereby be affected is exceedingly problematical. Harmonizing with this argument is the questionable effect upon the protein metabolism of glucose administration, as noted above.

*3. Effect of Ureteral Ligation upon Ketosis of Phlorhizinized Dogs.*

In Table III are given the preoperative and postoperative values of the ketonemia in those dogs which received phlorhizin both before and after operation. Because of the relatively large amounts of blood required for the estimation these values were not followed as frequently as the blood sugar and non-protein nitrogen.

TABLE III.

*Effect of Bilateral Ureteral Ligation upon Ketosis of Phlorhizinized Dogs.*

| Dog No. | Total acetone bodies of blood expressed as mg. of acetone per 100 cc. | |
|---|---|---|
| | Before operation. | After operation (hrs after operation shown in parentheses). |
| 36 | 23 1 | 7.8 (3), 6.0 (6), 5.2 (22), 0.0 (46) |
| 40 | 10 3 | 1.7 (3), 0.0 (22), 4.9 (30) |
| 41 | 9 0 | 0.0 (3), 1.0 (22), 0.0 (46), 2.6 (77) |
| 42 | 6.7 | 1.0 (3), 16.5 (22), 7.2 (46) |
| 43 | 10 2 | 4.5 (3), 0.7 (6), 7.0 (22), 1.8 (46), 0.0 (73) |
| 46 | 36.4 | 31.7 (3), 2.7 (8), 5.2 (22) |
| 48 | 23.8 | 24.7 (2), 3.2 (22), 0.0 (75) |
| 50 | 12.5 | 10.5 (2), 0.0 (22) |

TABLE IV.

*Effect of Phlorhizin upon Free Sugar of Liver and Muscle of Dogs with Ligated Ureters.*

| Dog. No. | Sugar per 100 gm. of tissue. | | Remarks. |
|---|---|---|---|
| | Muscle. | Liver. | |
| | *mg.* | *mg.* | |
| 36 | 72 | 218 | Phlorhizin before and after operation. |
| 42 | 61 | 352 | "        "        "        "        " |
| 43 | 40 | 340 | "        "        "        "        " |
| 44 | 62 | 405 | No phlorhizin. |

Deuel, Wilson, and Milhorat could not detect an "acetone breath" in their nephrectomized dogs after phlorhizin administration. In our experiments not only did we fail to detect acetone bodies in the blood when phlorhizin was first administered after ureteral ligation, but we observed that the existing ketosis of previously phlorhizinized animals diminished or disappeared more

or less promptly following operation. These findings, we believe, do not of necessity invoke a simultaneous oxidation of carbohydrate. While we find in the present experiments no additional support for our view as expressed elsewhere (11), we conceive that the mere accumulation of carbohydrate in the tissues may suffice for the normal and complete combustion of fat or other ketogenic substances.

### 4. Effect of Phlorhizin upon Free Sugar of Liver and Muscle of Dogs with Ligated Ureters.

The possibility that sugar might accumulate as such in the tissues of anuric, phlorhizinized dogs was investigated by analyses of tissues from several dogs of the series. The results (Table IV) are perhaps too few to be conclusive, but they do not suggest any difference in behavior as between phlorhizinized and non-phlorhizinized subjects.

### 5. Effect of Phlorhizin upon Glycogen Formation in Dogs with Ligated Ureters.

Complete data of the study of glycogen content of the animals of this series are given in Table I, while in Table II are shown the preoperative data of those dogs which were phlorhizinized prior to operation. While the average glycogen content of the liver after phlorhizin is distinctly higher than for control animals, individual variations in both groups prevent any generalization. No significant change in muscle glycogen appears after phlorhizin administration, unless it be in Dog 46. This animal was completely fasted for 9 days. For 3 days before operation the dog was phlorhizinized. On the day preceding the first injection of phlorhizin the dog was given an ice water bath and shivered for 2 hours at a low temperature. Adrenalin was administered during the 2nd day of the phlorhizin period, producing an excretion of only 5 gm. of "extra" sugar. In all probability, at the time of operation the liver of Dog 46 was practically glycogen-free. 54 hours after operation the liver contained 3.2 per cent glycogen, but the muscle glycogen was only 0.18 per cent, a value decidedly lower than observed in any other animal.

In no instance does it appear that the glycogen content of the

tissues at death might account substantially for carbohydrate produced or introduced subsequent to operation. Perhaps the data of Dog 46 afford the most valid basis for a calculation of this problem. Whether the muscle glycogen of this animal at death represents any accumulation following operation cannot be determined. While the dog was exceedingly weakened by the preoperative procedures to which it had been subjected, it is doubtful whether at any time the muscle glycogen was reduced much below the final value. On the other hand, it may be assumed that at operation the liver was essentially free of glycogen. Hence, in the liver we may account for about 13 gm. of carbohydrate which, presumably, originated from the postoperative protein metabolism. This is probably about 50 per cent of the total carbohydrate production during the 54 hour interval between operation and death.[4] The discrepancy between the amounts of carbohydrate available and the amounts recovered is larger in those animals which received glucose after operation (cf. Table I, Group 3-b). To what extent this discrepancy may represent carbohydrate oxidation is, we believe, still uncertain. With perfectly normal concentrations of glycogen in the livers of these animals, the possibility of fat synthesis is consistent with all of the phenomena observed, including an elevation in the respiratory quotient.

An observation surprising to us, at least, is the very substantial amounts of glycogen which may be present in the liver and muscles of animals after prolonged fasting and subjection to other presumptive deglycogenizing procedures such as exposure to cold, phlorhizinizing, adrenalin administration, anesthesia, and operation.

.

### SUMMARY.

In a series of eighteen dogs with ligated ureters, twelve of which were phlorhizinized, the sugar, non-protein nitrogen, and acetone bodies of the blood, and the glycogen of muscle and liver were studied. No conclusive influence of phlorhizin upon any of these values could be demonstrated. It is suggested that this result is not necessarily inconsistent with the view that the mechanism of phlorhizin action is not restricted to the kidneys.

Dogs which have fasted for as long as 9 or 10 days, during which

period and preliminary to ablation of kidney function they have been subjected to procedures generally relied upon to reduce glycogen reserves to minimal values, may still show substantially normal values for the glycogen content of muscles and liver.

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# THE NATURE OF BLOOD SUGAR. II.

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In a recent issue of this *Journal* (1) Folin criticized a paper by Somogyi and Kramer (2) who controverted some findings of Folin and Svedberg (3). The issue is this: Folin and Svedberg, having obtained lower values for fermentable sugar in blood by Folin's modified method (4) than by the Folin-Wu procedure, assumed the presence in blood of a fermentable sugar other than glucose "with so much lower reducing power than glucose that . . . it escaped determination, in whole or in part, when the more weakly alkaline copper reagent of Folin was used" (1). Somogyi and Kramer, on the contrary, found practically identical values for true sugar by several analytical methods. A justified exception could have been taken, however, as to the comparability of their results with those of Folin and Svedberg on the ground that the reagents employed by Somogyi and Kramer were far more alkaline than Folin's new reagent. Yet, additional comparative sugar determinations by four different procedures, including the two employed by Folin and Svedberg, seem substantially to bear out the previous findings of Somogyi and Kramer. Furthermore, we are able to furnish evidence, we believe, to show that the substance escaping oxidation in Folin's method is not a fermentable sugar but the complex of reducing non-sugars.

## Analytical Procedure.

In the comparative determinations of true (fermentable) sugar we made use of our zinc precipitation technique (5) which yields blood filtrates containing no appreciable amounts of non-fermentable reducing substances. Thus by a single determination sugar values are obtained representing at least as close an approach to true sugar values as the fermentation technique might afford.

TABLE I.

*Comparative Sugar Determinations in Zinc Filtrates of Human Blood by Four Analytical Procedures.*

| Case No. | Source. | Shaffer-Hartmann (modified). | Folin-Wu. | Folin. | Benedict. |
|---|---|---|---|---|---|
| | | Mg. sugar in 100 cc. blood. | | | |
| J. | Healthy man. | 70 | ˙ 69 | 67 | 62 |
| S. | "        " | 91 | 93 | 93 | 94 |
| G. | "        " | 76 | 76 | 71 | 76 |
| | Mixture of several samples. | 96 | 97 | 95 | 96 |
| | "     "     "     " | 101 | 104 | 101 | 103 |
| | "     "     "     " | 60 | 61 | 61 | 60 |
| 1011 | Non-diabetic patient. | 75 | 75 | 73 | 74 |
| 1032 | "         " | 70 | 68 | 66 | 68 |
| 1047 | "         " | 67 | 65 | 64 | 65 |
| 1063 | "         " | 100 | 96 | 97 | 99 |
| 1068 | "         " | 82 | 83 | 83 | 83 |
| 1069 | "         " | 91 | 91 | 89 | 95 |
| 1071 | "         " | 75 | 75 | 75 | 75 |
| 1072 | Fasting. | 67 | 67 | 66 | 64 |
| | After 100 gm. glucose. | | | | |
| | 30 min. | 126 | 126 | 125 | 124 |
| | 1 hr. | 145 | 148 | 142 | 143 |
| | 2 hrs. | 105 | 106 | 102 | 106 |
| | 3  " | 96 | 94 | 91 | 92 |
| 1008 | Diabetic patient. | 189 | 190 | 180 | 190 |
| 1014 | "         " | 155 | 152 | 148 | 156 |
| 1019 | "         " | 146 | 141 | 142 | 137 |
| 1027 | "         " | 310 | 308 | 309 | 315 |
| 1028 | "         " | 314 | 308 | 312 | 319 |
| 1026 | "         " | 200 | 195 | 196 | 197 |
| 1033 | "         " | 105 | 107 | 105 | 108 |
| 1041 | "         " | 119 | 120 | 122 | 120 |
| 1042 | "         " | 167 | 162 | 161 | 165 |
| 1050 | "         " | 128 | 133 | 124 | 131 |
| 1053 | "         " | 109 | 110 | 107 | 110 |
| 1057 | "         " | 156 | 159 | 150 | 157 |
| 1058 | "         " | 143 | 141 | 138 | 138 |
| $F_1$ | "         " | 266 | 262 | 263 | 266 |
| $F_2$ | "         " | 234 | 239 | 220 | 244 |
| $F_3$ | "         " | 178 | 174 | 178 | 174 |
| 1104 | "         " | 187 | 183 | 181 | 182 |

In the Folin and Folin-Wu methods we have followed the authors' directions with meticulous care, with the added precaution that, in order to rule out errors arising from the lack of complete proportionality in color development, the standard sugar solutions were always so chosen as to agree with the sugar concentration of the blood filtrates within 2.5 mg. per cent (or 0.05 mg. per 2 cc.). To make this possible, colorimetric work was always preceded by titrimetric determinations.

In the Shaffer-Hartmann method an improved copper reagent was used, made up of 7.5 gm. of copper sulfate, 20 gm. of sodium carbonate, 25 gm. of sodium bicarbonate, 25 gm. of Rochelle salt, 5 gm. of potassium iodide, and 10 cc. of $N$ potassium iodate per liter. Details concerning this reagent will be given in another paper.

### Sugar Determinations.

Comparative sugar determinations were performed by three colorimetric procedures: the Folin-Wu, the Folin, and the Benedict (6) methods, and in addition by the modified Shaffer-Hartmann method. As stated above, true sugar values were obtained by direct determinations in zinc filtrates. For the colorimetric determinations, since the copper reagents involved possess no buffer effect, the filtrates had to be neutralized by carbonate-free sodium hydroxide, with phenolphthalein as indicator.

The results, recorded in Table I, show that the four analytical procedures give identical sugar values within the rather narrow limits of experimental errors. The new Folin reagent yields in some cases slightly lower results than the other three, but the difference is conspicuous in a single case (Case $F_2$). The close agreement of the values of thirty-five determinations justifies the conclusion that, on the whole, Folin's new reagent gives the same values for true (fermentable) sugar as the original Folin-Wu method and the other two methods employed in this work, and, for that matter, as any other adequate method.

### Non-Fermentable Reducing Substances Escape Oxidation in Folin Method.

In apparent contradiction to the foregoing, we can corroborate the observation of Folin and Svedberg that Folin's procedure

TABLE II.

*Showing That in Folin's Method Reducing Non-Sugars Escape Oxidation if Sufficient Unoxidized Glucose Is Present.*

| Case No. | Time of heating. | Reduction values per 100 cc. blood, in terms of glucose. | | | | | Error. |
|---|---|---|---|---|---|---|---|
| | | Residual reduction. | Added glucose. | Total reduction. | | | |
| | | | | To be expected. | Found. | | |
| | *min.* | *mg.* | *mg.* | *mg.* | *mg.* | | *mg.* |
| 1 a | 10 | 12 | 75 | 87 | 80 | | −7 |
| | 20 | 13 | 75 | 88 | 90 | | +2 |
| 1 b | 10 | 12 | 200 | 212 | 198 | | −14 |
| | 20 | 13 | 200 | 213 | 213 | | 0 . |
| 2 | 10 | 9 | 100 | 109 | 102 | | −7 |
| | 20 | 11 | 100 | 111 | 114 | | +3 |
| 3 a | 10 | 10 | 50 | 60 | 61 | | +1 |
| | 20 | 14 | 50 | 64 | 66 | | +2 |
| 3 b | 10 | 10 | 200 | 210 | 189 | | −21 |
| | 20 | 14 | 200 | 214 | 214 | | 0 |
| 4 a | 10 | 8 | 75 | 83 | 80 | | −3 |
| | 20 | 9 | 75 | 84 | 88 | | +4 |
| 4 b | 10 | 8 | 200 | 208 | 197 | | −11 |
| | 15 | 9 | 200 | 209 | 206 | | −3 |
| 5 a | 10 | 8 | 50 | 58 | 56 | | −2 |
| | 20 | 9 | 50 | 59 | 59 | | 0 |
| 5 b | 10 | 8 | 200 | 208 | 198 | | −10 |
| | 20 | 9 | 200 | 209 | 206 | | −3 |
| Folin-Wu method. | | | | | | | |
| 5 a | 6 | 18 | 50 | 68 | 69 | | +1 |
| 5 b | 6 | 18 | 200 | 218 | 218 | | 0 |
| Shaffer-Hartmann method. | | | | | | | |
| 5 a | 15 | 15 | 50 | 65 | 65 | | 0 |
| 5 b | 15 | 15 | 200 | 215 | 219 | | +4 |

yields lower values for fermentable blood sugar than the Folin-Wu method (and, we may add, than the other two methods employed in this work), when the determination is carried out in *tungstic acid filtrates*, by deducting the residual reduction from the total reduction. Evidently some reducing substance does escape oxidation in Folin's procedure; some reducing substance which, as we have seen, is obviously absent from zinc filtrates but is present in tungstic acid filtrates. The assumption that it may be the non-sugar reducing substances (residual reduction) that are involved in this process, is supported by Benedict's interesting observation that "in the presence of glucose the residual reducing 'material is without effect" upon the reduction values when his new reagent is employed (7). This prompted an examination of Folin's method in this direction.

Tungstic acid filtrates were treated with washed yeast (8), and the reduction values of the fermented filtrates were determined before and after the addition of known amounts of pure glucose. The results, presented in Table II, were obtained by a simple procedure, executed, for example, in Case 1a as follows: A substantial sample of blood (about 20 cc.) was deproteinized by tungstic acid at 1:5 dilution. The filtrate was fermented, and after neutralization 1 cc. portions of it were measured into two Folin-Wu tubes. To one of the tubes, serving for the determination of the residual reduction, 1 cc. of water, to the other 1 cc. of a 0.015 per cent glucose solution were added; 1 cc. of the same glucose solution plus 1 cc. of water furnished the standard for the latter, while a tube with 2 cc. of a 0.001 per cent glucose solution was used as standard for the residual reduction. After the addition of Folin's reagent, the entire set, supported by a rack as employed in the Shaffer-Hartmann method, was placed in boiling water for 10 minutes. Subsequently, an identical set was heated for 20 minutes.

From Table II the following information may be obtained: (1) Folin's method yields too low reduction values in tungstic acid filtrates even when glucose is the sole fermentable substance present. (2) Too low reduction values occur only if the duration of heating does not exceed 10 minutes; upon more prolonged heating (15 or 20 minutes) both the glucose and the non-fermentable reducing substances are fully oxidized. (3) Even within 10 minutes the non-fermentable reducing substances are nearly

completely oxidized in the absence of sugar or if the sugar present
is not in excess of 50 to 60 mg. per cent of fermentable blood sugar.
With rising sugar concentrations a fraction of the reducing sub-
stances remains unoxidized, until at hyperglycemic sugar levels
this fraction is equal to the residual reduction.

These facts suggest the inference that glucose and non-ferment-
able reducing substances in tungstic acid filtrates are competing,
so to speak, for oxygen, glucose taking the precedence and thereby
inhibiting the oxidation of the non-sugars.   In Folin's procedure
16 to 20 per cent of the glucose (or more correctly, its cleavage
products) are always left unoxidized after 10 minutes' heating, and
if the initial sugar concentration has been high, as in diabetic blood, '
the unoxidized fraction of it suffices completely to protect the non-
sugars from being oxidized in 10 minutes.   At lower initial sugar
concentrations the protective effect is but partial and becomes
imperceptible at the lowest normal levels of blood sugar.   The
oxidation of the non-fermentable reducing substances is not in-
hibited by sugar if the heating is prolonged to 20 minutes, since at
this stage but a very small fraction of the sugar is left unoxidized.

Two examples given at the end of Table II show that in the Folin-
Wu and the Shaffer-Hartmann methods the reducing non-sugars
are completely oxidized regardless of the amount of the sugar
present.   This is in part due to the fact that in the Folin-Wu pro-
cedure only 6 to 8 per cent of the glucose is left unoxidized at the
end of 6 minutes heating, and almost none after 15 minutes in
the Shaffer-Hartmann method.   But there is an additional factor
affecting the process; namely, the degree of alkalinity.   If the
reaction is interrupted in the Folin-Wu procedure after less than
6 minutes heating, so that the oxidation is stopped about 20 per
cent short of completeness, no inhibition in the oxidation of the
non-sugars can be established.   With the Shaffer-Hartmann re-
agent employed in this work, on the other hand, we were able to
demonstrate the protective effect of glucose if the heating was
interrupted after 6 minutes, at which stage 16 to 18 per cent of
the glucose is still unoxidized.   A marked inhibition in the oxida-
tion of the non-sugars could be observed, however, only at a
sugar concentration about twice as high as suffices to produce the
like effect in Folin's procedure.   In other words, low alkalinity
favors the preferential oxidation of glucose.

The original blood sugar shows the same behavior as the pure glucose added to fermented blood filtrates. Accordingly, in the work of Folin and Svedberg the original Folin-Wu method gave the more correct values for fermentable sugar, while in the new Folin procedure, the deduction of the non-fermentable reducing substances from the total reduction led to erroneous results. The error is greater in hyperglycemic specimens, and this gave occasion to Folin and Svedberg to point out the greater discrepancies they had observed in diabetic than in non-diabetic cases.

In conclusion we may add a few words in reply to Folin's critical remarks. (1) The first objection voiced by Folin is that Somogyi and Kramer failed to take into account the autoreduction of the reagent employed in their colorimetric work. This objection is wholly without basis, since after a preliminary titrimetric determination, in subsequent colorimetric work standards were chosen to approximate closely the sugar content of the blood filtrates. It is obvious that in such a procedure the autoreductions in standard and blood filtrate cancel one another. (2) A second objection concerns the use of *open* test-tubes by Somogyi and Kramer. It is unfortunate that Folin overlooked the following statement of Somogyi and Kramer: "we employ ordinary test-tubes, covered with glass bulbs." As in the Shaffer-Hartmann method, the tubes are held in place on copper racks while being heated in a water bath, and it can be observed that condensed vapor soon forms an excellent seal between the cover and the mouth of the tube, thus excluding convection currents of air before any appreciable reduction had occurred. (3) Folin believes "as a matter of fact" that our results by the ferricyanide method are not "entirely reasonable" because our figures "are certainly not the kind of figures which should be obtained by the Hagedorn-Jensen method." They are not, and cannot possibly be. Somogyi and Kramer made their determinations on Folin-Wu (tungstic acid) filtrates, while Hagedorn and Jensen precipitated the blood proteins by heat coagulation in the presence of zinc hydroxide. Just the observation of the difference, to which Folin takes exception, led the writer to recognize the fact that the Hagedorn-Jensen precipitation technique leaves only about half as much of non-fermentable reducing substances in the filtrate as the tungstic acid technique.

The author acknowledges the helpfulness in this work of much valuable information he derived from his collaboration with Dr. P. A. Shaffer in studies of chemical processes involved in sugar determinations.

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# DETERMINATION OF SUGAR IN BLOOD.

By STANLEY R. BENEDICT.

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In recent numbers of this *Journal* (1, 2) two criticisms have appeared of the present writer's latest method (3) for the determination of blood sugar. The one of these by West, Scharles, and Peterson (1) is based wholly upon theoretical objections to a procedure which they have apparently not employed for even a single determination. Condemnation based on what *may* be the shortcomings of a method need not be taken too seriously. We shall therefore not discuss the points raised in their communication. The three detailed alternative procedures for blood sugar determinations suggested by them may explain this theoretical attack against the only method so far proposed which will give apparently accurate and minimal sugar values when applied directly to tungstic acid blood filtrates.

The criticism of the present writer's blood sugar method presented by Everett (2) appears at first glance to be a detailed and careful study of the procedures involved. Even a cursory examination of the actual data offered serves, however, to indicate that Everett's examination of the method was superficial and that he did not present findings related to the method as commonly employed. The essential criticism raised by Everett is that the present writer's method does not give true blood sugar values because of rapid and unequal fading in color of the unknown solution in comparison with the standard. To a considerable extent Everett's criticism resembles that of West and collaborators referred to above in that Everett fails to report any figures obtained through proper use of the method which he criticizes. The basis for Everett's criticism rests chiefly upon the results recorded in his Table III which is herewith reproduced. As a result of the

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figures given in this table, Everett states, "It may be argued that errors would be small if readings were made at once, but there remains the uncertainty of how much fading occurs before it is possible to compare the solutions in a colorimeter. . " Apparently Everett preferred not to attempt to settle this question in regard to the possible fading during the first 10 minutes after the color reagent is added. Of twelve blood samples for which figures are reported in his Table III, there is not one instance in which the reading was made less than 5 minutes after dilution and

*Everett's Table III*

The results are expressed in mg per cent

| Time after dilut-ing | Sample No | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *min* | | | | | | | | | | | | |
| 5 | 76 5 | 90 5 | 75 5 | 53 5 | 74 5 | 72 5 | 74 | 79 | 71 | | 125 | 72 5 |
| 10 | 72 | | | | | | | | 70 | 66 | 119 5 | |
| 15 | | | | | | | | | 68 5 | | | |
| 25 | | 84 5 | | | | | | | | | | 66 5 |
| 30 | | | | | | 62 | | | | | | |
| 35 | 68 | | 68 | 50 | 68 | | 65 5 | 72 | | | | |
| 40 | 61 | 80 | | | | | | | 63 5 | | 104 | |
| 50 | | | | | | | | | | 57 5 | | |
| 60 | | | | 48 | 67 5 | | | | | | | |

Sample 8 was plasma of Sample 9

Sample 9 without sulfite in reagent; 73 mg per cent at 10 minutes, 73 mg per cent at 40 minutes

Sample 10 without sulfite in reagent; 73 mg per cent at 5 minutes, 73 mg per cent at 50 minutes.

there are only three instances in which the figure obtained 5 minutes after dilution is compared with that obtained 10 minutes after dilution. Only the changes taking place in the readings during the first 10 minutes are likely to be of any practical significance, and one inspecting Everett's Table III might wonder why he omitted practically all of the readings which would let the reader judge what the practical shortcomings of the method might amount to.

In Table I are recorded readings made by the present writer

upon ten samples of blood.    These readings were made at once and at 1 minute intervals during the first 10 minutes after the addition of the color reagent and dilution.    A similar study of the question of the alleged more rapid fading of the unknown may of course be made readily by anyone interested.    Table I shows that where reasonable care is used when employing the writer's new copper reagent, inaccurate results will not arise due to unequal

TABLE I.

*Showing That the Low Results for Blood Sugar Obtained with Benedict's Reagent Are Not Due to Unequal Fading in Color between Standard and Unknown, Providing the Determinations Are Properly Made.*

The results represent readings in mm. against a standard solution set at 15 mm.

| Time after diluting. | Source and No. of blood sample. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pig. | | | | Beef. | Human. | | | | |
| | 1 | 2 | 3 | 4* | 5 | 6 | 7 | 8 | 9 | 10 |
| min. | | | | | | | | | | |
| 0 | 21.0 | 22.7 | 20.9 | 15.0 | 23.1 | 5.4 | 21.7 | 14.5 | 20.3 | 18.7 |
| 1 | 20.9 | 22.2 | 21.0 | 14.7 | 22.9 | 5.5 | 21.4 | 14.3 | 20.0 | 18.7 |
| 2 | 21.0 | 22.3 | 20.7 | 14.8 | 23.2 | 5.4 | 21.5 | 14.5 | 20.2 | 18.8 |
| 3 | 20.7 | 22.4 | 20.9 | 14.7 | 23.0 | 5.2 | 21.4 | 14.4 | 20.0 | 18.6 |
| 4 | 20.9 | 22.5 | 20.6 | 14.8 | 22.8 | 5.1 | 21.6 | 14.5 | 20.0 | 18.6 |
| 5 | 20.8 | 22.5 | 20.5 | 14.9 | 22.8 | 5.1 | 21.8 | 14.6 | 20.2 | 18.8 |
| 6 | 20.5 | 22.8 | 20.7 | 15.0 | 23.0 | 5.0 | 21.7 | 14.4 | 20.0 | 18.8 |
| 7 | 20.7 | 23.0 | 20.6 | 15.1 | 23.1 | 5.1 | 21.9 | 14.3 | 19.9 | 18.7 |
| 8 | 20.7 | 23.1 | 20.7 | 15.0 | 22.9 | 5.0 | 22.0 | 14.5 | 20.0 | 18.6 |
| 9 | 20.9 | 22.9 | 20.8 | 15.2 | 22.9 | 5.1 | 22.1 | 14.5 | 19.9 | 18.8 |
| 10 | 20.8 | 23.3 | 20.9 | 15.1 | 23.2 | 5.1 | 22.0 | 14.4 | 20.1 | 18.9 |

\* Blood diluted 1:5 instead of the usual 1:10.

fading between standard and unknown, and from Everett's Table III one might infer that only some two out of twelve samples of blood would show any appreciable loss in sugar value as late as 25 minutes after dilution.

Table V in Everett's paper shows that where readings are made with any reasonable degree of promptness after dilution, the recovery of sugar added to fermented blood filtrates is better by the present writer's method than by the Folin procedure with which it is compared.

Everett has stressed the use of the Folin color reagent in connection with the present writer's method though the reagent which we suggested is more readily prepared than is the Folin reagent. In the preparation of the color reagent care must be taken to use molybdic acid which is free from ammonia. Unless this is done, there may be a difference in shade of color between standard and unknown and there may also occur a difference in the rate of fading between the two solutions.

In conclusion we may state that the work of Everett may be of value to some in drawing attention to the fact that in the use of the present writer's new copper method for blood sugar determination, the solutions should not stand around the laboratory for long periods of time before the readings are made. This finding is, however, equally true of most of the commonly used colorimetric methods.

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# THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

## III. CONCERNING PHTHIOIC ACID. PREPARATION AND PROPERTIES OF PHTHIOIC ACID.*

BY R. J. ANDERSON.

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### INTRODUCTION.

Some time ago we described the preparation of a phosphatide (1) from the tubercle bacilli, Strain H-37, and reported a preliminary study of the composition of this substance (2). Subsequent investigations have proved that the phosphatide possesses unusually interesting and important properties. Sabin and Doan (3) observed that intraperitoneal injections of an aqueous suspension of the substance into normal rabbits caused a large increase in monocytes, epithelioid and giant cells and that the injections were followed by the development of massive typical tubercular tissue in the peritoneal cavity.

The study of the cleavage products (2) of the phosphatide indicated a marked difference in composition from the ordinary compounds which are designated by the name of phosphatides. The substance from tubercle bacilli contained only a very small amount of nitrogen in the form of ammonia and it contained about 33 per cent of water-soluble constituents consisting of carbohydrates and glycerophosphoric acid. The total fatty acids obtained on hydrolysis amounted to 67 per cent and among these acids we identified palmitic and oleic acids. We also found that the substance contained a new saturated fatty acid of high molecular weight which was optically active. As judged by the composition and molecular

weight, the acid contained twenty or more carbon atoms in its molecule.   The crude acid was probably a mixture of at least two higher saturated fatty acids because it could be separated into two fractions by means of its salt with benzylpseudothiourea, one of which was optically inactive while the other possessed a higher optical rotation as well as a higher molecular weight than the original material.

In considering the cause of the biological activity of the phosphatide the idea suggested itself that the optically active liquid saturated fatty acid might be the active principle.   It was believed that the peculiar physical properties of the acid might cause unusual physiological reactions.   At any rate this acid seemed to offer a better possibility than any of the other cleavage products. A larger quantity of the phosphatide was therefore hydrolyzed and the liquid saturated fatty acid was isolated and submitted to Dr. Sabin for physiological experiments.

The results of these studies were reported by Sabin, Doan, and Forkner (4) to the effect that the acid gave practically identical reactions to that of the phosphatide; *i.e.*, it stimulated the proliferation of monocytes and epithelioid cells and caused the formation of massive artificial tubercular tissue.

A number of different samples of this acid have been isolated and tested and all of these preparations have shown similar biological properties.   It is evident therefore, that the active principle of the phosphatide is associated with this new fatty acid and is always isolated with this fraction.

The lipoids of the tubercle bacilli have been regarded as possessing important biological properties for a long time.   Earlier observations by other investigators (5) have indicated that various mixtures of lipoids give reactions of similar nature to those reported by Sabin and Doan.   It is felt, however, that a distinct advance has been made at this time because the biological activity has been traced to a much smaller and more homogeneous fraction than has ever been done before.

Although the new fatty acid has not yet been obtained in pure form from the phosphatide the evidence regarding the activity appears to be very convincing.   More highly purified acids prepared from other fractions of the bacillary fat have been obtained and will be described shortly.   In order to indicate the relation of

this acid to tuberculosis we wish to designate it by the name phthioic acid.

Phthioic acid can only be separated from the mixed fatty acids by the complicated and laborious process outlined below: The mixed fatty acids are converted into the lead soaps and the latter are extracted with ether. After decomposition of the ether-insoluble lead soaps crude palmitic acid is obtained. The ether-soluble lead soaps on the other hand yield liquid acids containing oleic acid and phthioic acid and these products cannot be separated by any of the ordinary methods. However, by catalytic reduction, the unsaturated acid is converted into stearic acid and by repeating the lead soap-ether treatment only the lead salts of phthioic acid go into solution. By decomposing the lead salts with dilute hydrochloric acid the free phthioic acid can be isolated.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Hydrolysis of the Phosphatide.*[1]</div>

The purified Fraction A-4 of the phosphatide described in a former paper (2) was used in this experiment. The substance, 35.9 gm., was rubbed to a fine suspension in 500 cc. of water and 30 gm. of concentrated sulfuric acid were added. A heavy coagulum separated on warming and the mixture was boiled under a reflux condenser until the coagulum had been converted into an oily layer. No solid particles were visible after refluxing for 6 hours and after boiling for 8.5 hours the mixture was allowed to cool.

The fatty acids were extracted by shaking the hydrolysis mixture with three portions of ether. The ethereal extracts were combined and washed with water, the washings being added to the main aqueous solution. The washed ethereal solution was filtered and the ether was distilled. The residue consisting of crude fatty acids formed a brown crystalline mass which after it had been dried in a vacuum desiccator weighed 23.9 gm.

The aqueous solution containing the water-soluble constituents was in this case not further examined, but we know from

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[1] Throughout this work carbon dioxide was used to displace air in solvents, utensils, etc.

previous work that the solution contains carbohydrates and glycerophosphoric acid.

### Separation of Mixed Fatty Acids.

The mixed fatty acids were dissolved in 50 cc. of alcohol, neutralized with potassium hydroxide, and diluted with about 300 cc. of water. To this solution were added 300 cc. of 10 per cent lead acetate. The lead soaps were filtered on a Buchner funnel, washed with water, dried *in vacuo* over sulfuric acid, and then extracted with 300 cc. of ether. The ether-insoluble lead soaps were filtered off and washed with ether. The ether-soluble lead soaps were decomposed by shaking the ethereal solution with dilute hydrochloric acid and the lead chloride and hydrochloric acid were removed by washing with water. The ethereal solution was then filtered and the ether was distilled. The residue was a brown oil which after it had been dried in an atmosphere of carbon dioxide weighed 14.3 gm.

The ether-insoluble lead soaps were decomposed in a similar manner by shaking with ether and dilute hydrochloric acid, yielding 8.6 gm. of palmitic acid.

### Separation of Liquid Saturated Fatty Acids from Unsaturated Acids after Catalytic Reduction.

It has been shown in an earlier paper (2) that the liquid fatty acids obtained from the ether-soluble lead salts had an iodine number of about 18, indicating the presence of a large proportion of a liquid saturated fatty acid. In order to separate these liquid acids it is necessary first to convert the unsaturated acid by catalytic reduction into stearic acid. The latter can then be removed by repeating the lead soap-ether treatment.

The crude liquid acids mentioned above were mixed with 100 cc. of alcohol, when a small amount of insoluble matter, probably wax which had not been removed completely from the phosphatide, remained. The solution was cooled in ice water for some time after which the insoluble material was filtered and washed with alcohol. The filtrate and washings were united and treated with norit. The faintly yellowish solution was mixed with 0.3 gm. of platinum oxide prepared according to Voorhees and Adams (6)

and shaken with hydrogen under 15 cm. pressure until absorption ceased. The mixture was then allowed to stand overnight, when the catalyst separated completely. The platinum black was filtered off and the filtrate was concentrated by distillation to about 50 cc., neutralized with potassium hydroxide, diluted with water, and precipitated by adding an excess of lead acetate solution. The lead soaps, which formed a pasty mass, were washed several times with water without being removed from the flask and they were then treated with 200 cc. of ether. The insoluble lead salts were filtered off, washed with ether, and finally decomposed by shaking with ether and dilute hydrochloric acid. The crude stearic acid obtained in this manner weighed 4.3 gm.

The ethereal solution of the lead salt of phthioic acid was decomposed by shaking with dilute hydrochloric acid. After removal of the lead chloride and hydrochloric acid by washing with water, the solution was filtered and the ether was distilled. The faintly yellow oil which remained, after drying *in vacuo* over sulfuric acid, weighed 8.2 gm.

The amounts of the different fatty acids that were obtained are as follows:

*Hydrolysis of Phosphatide and Separation of Fatty Acids.*

Phosphatide taken, 35.9 gm.

|  | gm. | per cent |
|---|---|---|
| Total mixed fatty acids obtained | 23.9 or | 66.5 |
| Crude palmitic acid | 8.6 " | 23.9 |
| Alcohol-insoluble wax | 1.4 " | 3.9 |
| Stearic acid by reduction | 4.3 " | 11.9 |
| Phthioic acid | 8.2 " | 22.8 |

The loss in fatty acids during the various operations amounted to 1.4 gm. or about 3.9 per cent.

## Properties of Phthioic Acid.

The crude acid is obtained as a faintly yellowish oil. It can be decolorized by treating the ethereal or alcoholic solution with norit, yielding a practically colorless product which is liquid at room temperature. When cooled in ice water it forms a white solid which liquifies on warming to about 10°. The acid is saturated and in chloroform solution it does not decolorize bromine.

It is dextrorotatory and the specific rotation, in alcoholic solution, is about $+1.5°$ to $+1.6°$.   It is miscible in all proportions with alcohol and other organic solvents, but it is insoluble in water. The potassium and sodium salts are easily soluble in alcohol and in water.   The silver salt separates as a white amorphous precipitate on addition of silver nitrate to an alcoholic solution of the potassium salt.   The silver salt is very sensitive to light and very slightly soluble in water or organic solvents and it has not been obtained in crystalline form.   The acid forms a beautifully crystalline salt with benzylpseudothiourea (2).

*Rotation.*—1.1509 gm of substance dissolved in alcohol and made up to 10 cc. gave a reading of $+0.17°$, hence $[\alpha]_D^{20} = +1.47°$. Another preparation showed the specific rotation of $+1.66°$.

*Titration.*—0 3506 gm. of substance dissolved in 50 cc. of neutral alcohol required 11.19 cc. of 0.1 N alcoholic potassium hydroxide, with phenolphthalein as indicator.   Molecular weight found, 313. Other preparations gave values of 313, 306, 309, and 313.

For analysis the substance was dried at $61°$ *in vacuo* over phosphorus pentoxide but there was no loss in weight.

0 1162 gm. substance· 0 1348 gm  $H_2O$ and 0 3251 gm  $CO_2$.
Found    C 76 30, H 12 98 per cent
Other preparations gave C 76 17, 76 22; H 12 88, 12 84 per cent

In conclusion it is a pleasure to acknowledge the helpful cooperation extended by Dr. William Charles White, Professor Treat B. Johnson, Dr. F. R. Sabin, Dr. C. A. Doan, H. K. Mulford and Company, and Parke, Davis and Company.

### SUMMARY.

A method is described for the separation from the fatty acids obtained on hydrolyzing the phosphatide from the human type of tubercle bacilli, Strain H-37, of a new saturated fatty acid, named phthioic acid.

Phthioic acid is optically active and while the formula has not yet been determined definitely the composition and molecular weight indicate that it must contain at least twenty carbon atoms in its molecule.   Phthioic acid possesses important biological properties.

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# THE CONFIGURATIONAL RELATIONSHIP OF 2-METHYL-HEPTANOL-(6) TO LACTIC ACID.

## WITH A NOTE ON THE EFFECT OF UNSATURATION ON OPTICAL ACTIVITY.

BY P. A. LEVENE AND H. L. HALLER.

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The present communication is the first of a series which will deal with the configurational relationship of branched chain secondary carbinols with lactic acid. In the present case dextro-2-methylheptanol-(6) is correlated with dextro-lactic acid. The method employed in correlating the configurations of the two substances is one which has been used successfully on other occasions.[1] An unsaturated derivative, 2-methylhepten-(2)-ol-(6), is used as a connecting link between the substance of unknown configuration and one whose configuration is known. The details are given in Figures I to VI.



| I. | II. | III. | IV. | V. | VI. |
|---|---|---|---|---|---|
| Dextro. | Dextro, +18.0 in water. | Levo, −7 7 in ether. | Dextro, +19.0 in ether, +16.1 homogeneous. | Dextro, +13.0 in ether, +10.3 homogeneous. | Dextro, +9.76 homogeneous.[2] |
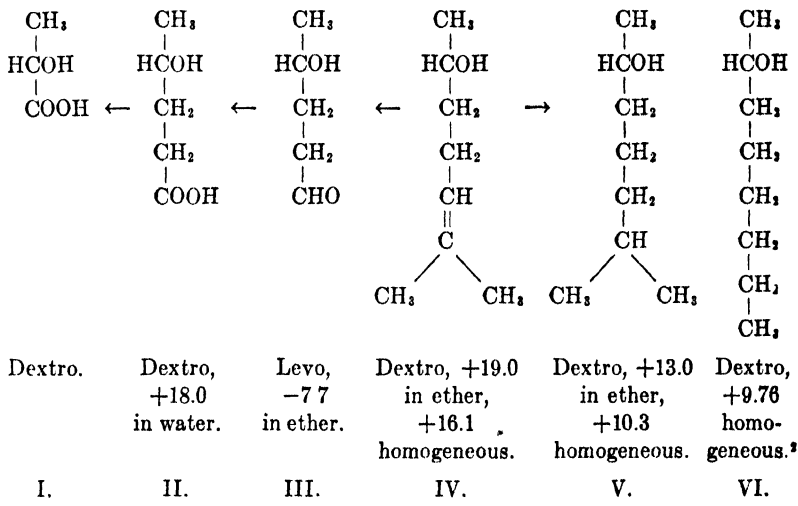
[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928); **79**, 475 (1928).

[2] Maximum rotation determined by Pickard and Kenyon. Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 49 (1911).

From these figures it is seen that in the present case the substitution of a normal for a branched chain did not alter the direction of rotation of the carbinol. It is interesting in this connection to compare the rotation of the three related substances, namely, of the normal hexylmethyl carbinol, of the 2-methylheptanol-(6), and of the unsaturated 2-methylhepten-(2)-ol-(6). The respective molecular rotations are: $+12.7°$, $+13.4°$, and $+22.4°$.[3] In the case of these three substances, the substitution of a branched chain radical for a normal chain enhances the value little, if at all, of the optical rotation of the "iso" alcohol as compared with that of the normal alcohol; the introduction of a double bond in the heavier radical brings about an exaltation of the optical rotation. It would be unwise, however, to conclude that either one of these factors, the double bond or the branched chain, has a constant effect on the optical rotation. The data already available regarding the influence of the double bond bring out the importance of another contributing factor, namely, the distance of the double bond from the asymmetric carbon atom.

From Figures IV, V, and VII to XI, it seems that exaltation of the rotation by a double bond is produced when the double bond is situated between carbon atoms (3) and (4) from the asymmetric carbon atom. It had been previously accepted that the effect of a double bond generally is an exaltation of the rotation of the parent saturated substance. Thus we find the statement of Pickard and Kenyon,[4] "—the effect of unsaturation is exhibited in the exaltation of rotatory power." Again the same statement appears in the later article of Kenyon and Snellgrove.[5] Rupe,[6] who studied the effect of unsaturation in the series of menthyl esters of saturated and unsaturated acids observed that as a general rule unsaturation produced an exaltation, the only exception being in the case of the ester of $\gamma,\delta$-hexenic acid. This ester showed a lower molecular rotation than the corresponding ester of hexonic acid. Thus neither the rule of Pickard and Ken-

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[3] It is not certain whether the last two values represent maximum rotations.

[4] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 47 (1911).

[5] Kenyon, J., and Snellgrove, D. R., *J. Chem. Soc.*, **127**, 1169 (1925).

[6] Rupe, H., *Ann. Chem.*, **327**, 157 (1903).

*Influence of Double Bond.*[7]

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $CH_3$ | $CH_3$ | $CH_2$ | $CH_3$ | $CH_2$ | $CH_3$ | $CH_2$ | $CH_3$ | $CH_3$ |
| $-CH_2$ | $-CH$ | $=CH$ | $-CH_2$ | $=CH$ | $-CH_2$ | $=CH$ | $-CH_2$ | $-CH_2$ |
| $-CH_2\ \uparrow$ | $=CH$ | $-CH_2$ | $-CH_2\ \uparrow$ | $-CH_2$ | $-CH_2\ \uparrow$ | $-CH_2$ | $-CH_2\ \uparrow$ | $-CH_2$ |
| $HOCH$ | $HOCH$ | $HOCH$ | $HOCH$ | $CH_2\ HOCH$ | $CH_2\ HOCH$ | $CH_2\ HOCH$ | $HOCH$ | $HOCH$ |
| $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $-CH_2$ | $CH$ |
| | | | | | | | $CH_3$ | $=CH_2$ |
| +7.95 | Slightly levo. +6.50 | +2.6 | +8.25 | +12.25 | +1.35 | +0.15 | +2.75 | −19.40 |
| VII.[8] | VII.[8] | VIII.[9] | IX.[10] | IX.[10] | X.[11] | X.[11] | XI.[12] | XI.[12] |

[7] All of the rotations are without solvent.

[8] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 703 (1929).

[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

[10] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

[11] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928).

[12] Unpublished observations.

yon nor that of Rupe holds in our series.    The difference between these three series and ours lies in the fact that in the former, the asymmetric carbon atom is connected to the unsaturated radical through an oxygen bridge, or the double bond in all members is at the same distance from the asymmetric carbon atom, whereas in our series, the asymmetric carbon atom and the double bond are located in the same carbon chain and the distance of the double bond from the asymmetric carbon atom is varied.

The general effect of the introduction of a branched chain will be discussed on another occasion when more experimental data will be available.

*The general conclusion from our work up to date is that the optical rotation of a substance is the resultant of the simultaneous action of several factors, of which three have been definitely pointed out.    These are: first, the respective masses of the radicals attached to the asymmetric carbon atom, second, the polarity of the groups, and third, the distances of the polar groups from the asymmetric carbon atom.*

### EXPERIMENTAL.

*2-Methylhepten-(2)-ol-(6)*.—The inactive carbinol was obtained on reduction of a commercial product of 2-methylhepten-(2)-one-(6) with sodium and absolute alcohol.

*Resolution of 2-Methylhepten-(2)-ol-(6)*.—The acid phthalate of the carbinol was prepared by heating a solution of 128 gm. of the carbinol and 148 gm. of phthalic anhydride in 250 cc. of dry pyridine on the steam bath for 1 hour.    The isolation and purification of the acid phthalate was carried out in the usual manner.[10]    The acid phthalate remained a thick syrup in spite of repeated attempts to crystallize it.

50 gm. of the acid phthalate were dissolved in 400 cc. of acetone, the solution was heated to boiling on the steam bath, and 85 gm. of brucine were added.    The hot solution was filtered and placed in the refrigerator.    The brucine salt soon crystallized.    It was filtered off and recrystallized several times from acetone.    The rotation of the brucine salt in absolute alcohol then was

$$[\alpha]_D^{24} = \frac{-0.31° \times 100}{1 \times 7.46} = -4.2°.$$

The brucine salt, obtained as described above, was decomposed with dilute hydrochloric acid and the acid phthalate isolated by the usual procedure. In ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 11.82° \times 100}{1 \times 19.6} = + 60.3°.$$

The acid phthalate, obtained as described above, was dissolved in an aqueous solution of sodium hydroxide (3 mols) and the solution was steam-distilled. The carbinol was isolated in the usual way. It distilled at 60–61°, p = 4 mm. It analyzed as follows:

3.975 mg. substance: 10.945 mg. $CO_2$ and 4.470 mg. $H_2O$.
$C_8H_{16}O$. Calculated. C 75.00, H 12.50.
Found. " 75.08, " 12.58.

In ether the carbinol had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 1.60° \times 100}{1 \times 8.4} = + 19.0°.$$

A carbinol obtained on decomposition of the mother liquors in the above resolution distilled at 87°, p = 22 mm. This carbinol without solvent had a rotation of $\alpha_D^{23} = -13.75°$, $l = 1$. In ether the rotation was

$$[\alpha]_D^{23} = \frac{- 3.20° \times 100}{1 \times 19.8} = - 16.2°.$$

An aliquot part of the ether solution was diluted with an equal volume of ether; the rotation then was

$$[\alpha]_D^{23} = \frac{- 1.65° \times 100}{1 \times 9.9} = - 16.7°.$$

In absolute alcohol the rotation of the carbinol was

$$[\alpha]_D^{23} = \frac{- 7.50° \times 100}{2 \times 24.9} = - 15.1°.$$

*α-Naphthylurethane of Dextro-2-Methylhepten-(2)-ol-(6).*—The urethane was prepared from a 2-methylhepten-(2)-ol-(6) having $[\alpha]_D^{20} = + 19.0°$ in ether, in the usual manner. Recrystallized from dilute alcohol it melted at 64–66°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.40 cc. 0.1 N HCl.
$C_{19}H_{23}O_2N$. Calculated. N 4.72.
Found. " 4.76.

In absolute alcohol the rotation of the urethane was

$$[\alpha]_D^{n} = \frac{+ 1.57° \times 100}{2 \times 2\,39} = + 32.8°.$$

*Levo-4-Hydroxyvaleric Aldehyde (Pentanal-(1)-ol-(4)).*—The carbinol, obtained as described above, was ozonized in glacial acetic acid, the procedure being essentially the same as that described by Helferich[13] for the ozonization of the inactive carbinol.

Into 17.5 gm. of dextro-2-methylhepten-(2)-ol-(6) ($[\alpha]_D^{24} = + 19.0°$ in ether) dissolved in 27 cc. of glacial acetic acid, a stream of ozonized oxygen was passed until the solution no longer decolorized bromine in glacial acetic acid. The remaining liquid was then diluted with ether. The decomposition of the ozonide and the isolation of the hydroxyaldehyde was then carried out in the same manner as described by Helferich. The hydroxyaldehyde distilled at 43–46°, p = 1 to 2 mm. It analyzed as follows:

4 870 mg. substance· 10 795 mg. $CO_2$ and 4 265 mg $H_2O$.
$C_5H_{10}O_2$.   Calculated.  C 58 82, H 9 80.
        Found.      " 60 44, " 9 75

In ether it had the following optical rotation.

$$[\alpha]_D^{B} = \frac{- 1.03° \times 100}{1 \times 13.2} = - 7.8°.$$

*Dextro-4-Hydroxyvaleric Acid.*—A mixture of 1.2 gm. of levo-4-hydroxyvaleric aldehyde ($[\alpha]_D^{23} = - 7.7°$ in ether), 2.5 gm. of silver oxide, and 40 cc. of water was heated to boiling under a reflux condenser for 20 minutes. The hot solution was filtered and concentrated under reduced pressure to a small volume. On addition of absolute alcohol the silver salt readily separated. It was filtered off and dried on a high vacuum pump. It analyzed as follows:

0.1008 gm. substance: 0.0490 gm. Ag.
        $C_5H_9O_3 \cdot Ag$.  Calculated.  Ag 47 99.
                Found      " 48.61.

0.15 gm. of silver salt was dissolved in water and the volume made up to 5.0 cc. The rotation was

$$[\alpha]_D^{n} = \frac{+ 0.32° \times 100}{2 \times 3.0} = + 5.4°.$$

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[13] Helferich, B., *Ber. chem. Ges.*, **52,** 1123 (1919).

To 4.2 cc. of the silver salt solution employed above was added 0.7 cc. of 1.0 N HCl. The rotation was observed immediately. For the free acid,

$$[\alpha]_D^{22} = \frac{+ 0.50° \times 100}{2 \times 1.33} = + 18.8°.$$

*Dextro-2-Methylheptanol-(6)*.—5 gm. of dextro-2-methylhepten-(2)-ol-(6) ($[\alpha]_D^{20} = +19.0°$ in ether) were dissolved in ether and reduced with hydrogen in the presence of colloidal palladium as catalyst. The absorption of hydrogen was slow but constant. Reduction was complete in 30 hours. The ether extract was dried over anhydrous potassium carbonate, the ether was removed, and the carbinol was distilled under reduced pressure. It distilled at 61–63°, p = 4 mm. It analyzed as follows:

3.430 mg. substance: 9.315 mg. $CO_2$ and 4.390 mg. $H_2O$.
$C_8H_{18}O$. Calculated. C 73.85, H 13.85.
Found. " 74.05, " 14.32.

In ether it had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 2.25° \times 100}{1 \times 17.3} = +13.0°.$$

*α-Naphthylurethane of Dextro-2-Methylheptanol-(6)*.—The urethane was prepared in the usual way. It was recrystallized from dilute alcohol. It melted at 75–77°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.35 cc. 0.1 N HCl.
$C_{19}H_{25}O_2N$. Calculated. N 4.68.
Found. " 4.69.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 0.82° \times 100}{2 \times 2.50} = + 16.4°.$$

# ON THE CONFIGURATIONAL RELATIONSHIP OF CHLOROSUCCINIC ACID TO CHLOROPROPIONIC AND TO LACTIC ACIDS.

BY P. A. LEVENE AND H. 'L. HALLER.

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The considerations which led up to the present investigation have been discussed in an earlier publication.[1] The principal object was to test by a second method the conclusions which had been reached earlier by Levene and Mikeska[2] regarding the configurational relationships of hydroxy and halogeno acids. The conclusions formulated earlier were the following:—(1) Dextro-2-hydroxypropionic (lactic) acid is configurationally related to dextro-2-chloropropionic acid. (2) Dextro-3-hydroxybutyric acid is configurationally related to *levo*-3-chlorobutyric acid. (3) Dextro-malic acid is configurationally related to *levo*-chlorosuccinic acid.

The conclusions of Levene and Mikeska have been tested regarding the first two pairs of acids by Levene and Haller by a different method and the new method led to the original conclusion. The same method has now been applied for testing the conclusions regarding the configurations of malic and chlorosuccinic acids.

The new method is based on the assumption that in aliphatic alcohols the substitution of the hydroxyl by a halogen atom proceeds without Walden inversion. The set of reactions employed for the solution of our problem is the following.

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[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **63**, 85 (1925); **70**, 365 (1926).

185

$$
\begin{array}{ccccc}
 & & CH_2 & CH_2 & \\
 & & \| & \| & \\
COOH & COOH & CH & CH & COOH \\
| & | & | & | & | \\
HCOH & \leftarrow \;\; HCOH & \leftarrow \;\; HCOH \;\; \rightarrow & HCCl & \rightarrow \;\; HCCl \\
| & | & | & | & | \\
CH_3 & CH_2 & CH_2 & CH_2 & CH_2 \\
 & | & | & | & | \\
 & COOH & CH & CH & COOH \\
 & & \| & \| & \\
 & & CH_2 & CH_2 & \\
\end{array}
$$

Levo.      Dextro.      Levo.      Dextro.      Levo.

$$
\downarrow
$$

$$
\begin{array}{c}
CH_3 \\
| \\
CH_2 \\
| \\
HCOH \\
| \\
CH_2 \\
| \\
CH_2 \\
| \\
CH_3 \\
\end{array}
$$
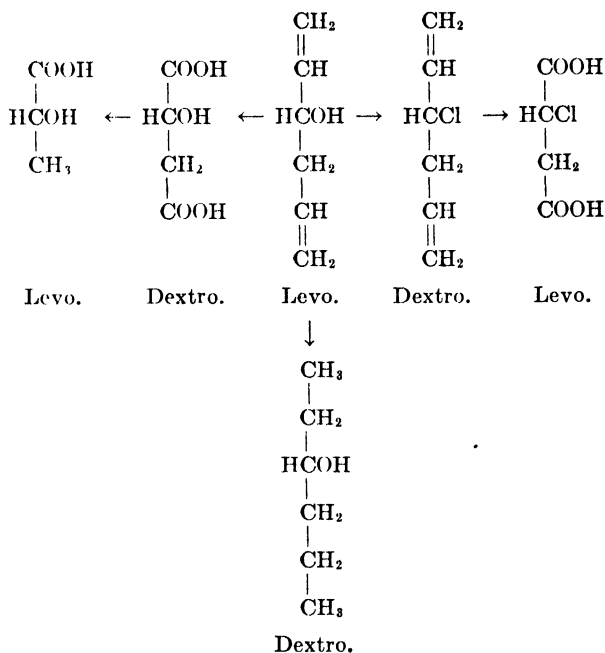
Dextro.

*Thus, the new test substantiates the conclusion previously reached by Levene and Mikeska to the effect that dextro-malic acid is configurationally related to levo-chlorosuccinic acid.*

Incidentally, it may be mentioned that the hexadienol which served as starting material for this investigation is a new substance. The method of its preparation is given in the experimental part.

### EXPERIMENTAL.

*Hexadiene-(1,5)-ol-(3) (Vinylallyl Carbinol).*—This carbinol[3] was obtained on condensation of acrolein and allyl bromide with zinc. It can also be prepared by the action of acrolein on allyl magnesium bromide. The crude product distilled at 125–135° at atmospheric pressure. It was purified through the acid

[3] We are indebted to Mr. R. E. Marker for assistance in the preparation of the carbinol.

phthalate. It then distilled at 133–134° at atmospheric pressure. $n_D^{25} = 1.4464$, $D_4^{25} = 0.8596$. It analyzed as follows:

4.305 mg. substance: 11.675 mg. $CO_2$ and 4.000 mg. $H_2O$.
$C_6H_{10}O$.    Calculated.    C 73.47, H 10.20.
                Found.    " 73.95, " 10.39.

*Resolution of Hexadiene-(1,5)-ol-(3).*—The acid phthalate of the carbinol was prepared by heating a solution of 107 gm. of the crude carbinol, 161 gm. of phthalic anhydride and 200 cc. of dry pyridine on the steam bath for 1 hour. After standing overnight at room temperature, the solution was cooled in an ice water bath, ice and ether were added, followed by 300 cc. of concentrated hydrochloric acid. The ether extract was washed with water and dried over sodium sulfate. The ether was removed under reduced pressure and the remaining syrup poured into a solution of 115 gm. of sodium carbonate in 1000 cc. of water. After standing for 1 hour at room temperature, the solution was extracted twice with ether, cooled, and acidified with 250 cc. of concentrated hydrochloric acid. The phthalate was extracted with chloroform, the chloroform extract was washed with water and dried over sodium sulfate. The chloroform was removed under reduced pressure and the remaining syrup was titrated and converted to the brucine salt.

236 gm. of the syrup, 60 per cent acid phthalate, were dissolved in 600 cc. of dry acetone, the solution heated to boiling and 290 gm. of brucine were added. The hot solution was filtered and placed in the refrigerator. On cooling with stirring, the brucine salt crystallized. It was filtered off and repeatedly recrystallized from dry acetone. The brucine salt was decomposed with dilute hydrochloric acid and the acid phthalate extracted with ether in the usual way. The phthalate was a thick syrup. In ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{+\,8.75° \times 100}{2 \times 24.3} = +\,18.0°.$$

The phthalate was dissolved in a solution of sodium hydroxide (3 mols) and the solution was steam-distilled. The carbinol was extracted with ether, and the ether extract was dried over anhydrous potassium carbonate. After removal of the ether,

the carbinol was distilled at atmospheric pressure. It distilled at 133–134°. It analyzed as follows:

5.105 mg. substance: 13.820 mg. $CO_2$ and 4.745 mg. $H_2O$.
$C_6H_{10}O$. Calculated. C 73.47, H 10.20.
Found. " 73.82, " 10.40.

The rotation without solvent was $\alpha_D^{24} = +16.0°$. $l = 1$ dm. In ether the rotation was

$$[\alpha]_D^{24} = \frac{+\ 1.45° \times 100}{1 \times 11.2} = +\ 12.9°.$$

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{24} = \frac{+\ 2.25° \times 100}{1 \times 10.8} = +\ 20.8°.$$

*Levo-3-Chloro-Hexadiene-(1, 5) (Vinylallyl Methyl Chloride).*—Into 5 gm. of phosphorus trichloride cooled in an ice water bath was dropped a solution of 10 gm. of dextro-hexadiene-(1,5)-ol-(3) ($[\alpha]_D^{24} = +20.8°$ in alcohol) and 1.5 cc. of dry pyridine. The reaction mixture was constantly shaken and after addition of the carbinol was warmed gently. After standing for 1 hour at room temperature, the chloride was distilled under reduced pressure. It analyzed as follows:

0.1358 gm. substance: 0.1510 gm. AgCl.
No. 1271. $C_6H_9Cl$. Calculated. Cl, 30.47.
Found. " 29.69.

In a 1 dm. tube without solvent $\alpha_D^{24} = -12.10°$. In ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{-\ 1.60° \times 100}{1 \times 12.1} = -\ 13.2°.$$

In absolute alcohol the rotation was

$$[\alpha]_D^{24} = \frac{-\ 1.95° \times 100}{2 \times 8.2} = -\ 11.9°.$$

*Dextro-Chlorosuccinic Acid.*—The chloride obtained as described above was ozonized in chloroform solution in 1 gm. lots. 1 gm. of the chloride (No. 1271) was dissolved in 40 cc. of chloroform and a stream of ozonized oxygen was passed into

the solution until it no longer decolorized bromine in glacial acetic acid. By this time a small quantity of a gelatinous precipitate adhered to the walls of the tube. The remaining chloroform was removed under reduced pressure and the ozonide poured into water and immediately treated with bromine. The reaction mixture was thoroughly shaken and then allowed to stand overnight with an excess of bromine. The remaining bromine was removed with a stream of air, the solution was thoroughly cooled and treated with silver sulfate until free of bromide ion, then with hydrogen sulfide to remove the excess silver. After saturating the solution with sodium sulfate, it was extracted with ether. The ether extract was dried over sodium sulfate. After removal of the ether, the remaining syrup was placed in the desiccator. After 2 days the crystals were filtered off. They were recrystallized from a mixture of ether and petroleum ether. The acid melted at 168–171°. It analyzed as follows:

7.235 mg. substance: 6.970 mg. AgCl.
$C_4H_5O_4Cl$. Calculated. Cl, 23.27.
Found. " 23.83.
12.078 mg. substance required 1.515 cc. 0.1 N NaOH.
Calculated. 1.580.

In ether containing 20 per cent alcohol the rotation was

$$[\alpha]_D^{24} = \frac{+\ 5.25° \times 100}{1 \times 11.0} = +\ 47.7°.$$

In water it had the following rotation.

$$[\alpha]_D^{24} \quad \frac{+\ 1.85° \times 100}{2 \times 4.9} \quad +\ 18.9°$$

*Dextro-Hexanol-(3) (Ethylpropyl Carbinol).*—4 gm. of levo-hexadiene-(1,5)-ol-(3) ($[\alpha]_D^{23} = -6.2°$ in ether) were reduced in ether solution with hydrogen in the presence of colloidal palladium as catalyst. Absorption of hydrogen was rapid and reduction was complete in 3 hours. The ether solution was dried over anhydrous potassium carbonate. After removal of the ether, the carbinol was distilled at atmospheric pressure.

It boiled at 128–130°. In a 1 dm. tube without solvent $\alpha_D^{23} = +1.20°$.

It analyzed as follows:

3.335 mg. substance: 9.155 mg. $CO_2$ and 3.795 mg. $H_2O$.

$C_5H_{14}O$.    Calculated.    C 70.59, H 13.72.
            Found.        " 70.58, " 13.21.

# OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL SIGNIFICANCE.

## IV. COMPARATIVE STUDY OF THE COMPLEXES OF CYSTEINE WITH THE METALS OF THE IRON GROUP.

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### INTRODUCTION.

Cysteine combines with iron to form complex compounds, which, according to Warburg, play the decisive rôle in the catalytic effect of iron in the oxidation to cystine. Besides iron, copper and manganese work as catalysts. These recent observations have given a new start to the old problem of oxidation .catalysis in the living organisms. However, in order to understand the chemical processes underlying this catalysis it is necessary to make a comparative study of the reaction of cysteine with other metals, which do not work as catalysts. Such a comparative study may give us information about the iron catalysis. With this idea in mind, the behavior of other metals of the iron group has been studied recently in Warburg's laboratory also, especially by Cremer (1), who found a compound of ferrous cysteine and of cobaltous cysteine with carbon monoxide. In the present paper, the complexes of cysteine with the metals of the iron group, Ni, Co, Fe, are to be subjected to a study, as far as it can be done, without preparing these complexes in pure condition, on the basis of color reactions, gas analytical and potentiometric methods..

### 1. Nickel.

The simplest case is that of cysteine and nickel. When a solution of cysteine buffered by phosphate or borate to a pH about 7.3 to 8.5 is mixed with nickel nitrate, a Bordeaux red color arises,

which is independent of the presence of oxygen.  This color is
rather deep in sufficient concentration of the reagents, but weak in
dilute solution when compared with some of the other color reac-
tions which are to be described in this paper.  The color is very
stable.  The precipitate of the basic nickel salt, which arises in a
solution of a nickel salt on addition of an alkali, is dissolved by
cysteine, giving rise to the soluble colored complex.  Nickel salts
do not work as catalysts for the oxidation of cysteine by oxygen
or by a reducible dye, nor do they influence when present in small
amounts the catalytic effect of small amounts of iron salts.  Krebs
(2) has recently shown that nickel is a powerful catalyst for the
oxidation of $H_2S$ by air.  As this is not the case for the oxidation
of cysteine, it becomes evident that the mechanism of oxidation
of $H_2S$ is different from that of cysteine.  All metals which work as
catalysts for cysteine possess the property of easily changing their
valency: Fe, Cu, Mn.  This is not true for nickel which both in the
free ionic and in the complex form is known practically only in
the nickelous state.  The catalysis in the case of $H_2S$ does not seem
to involve the cyclic change of valency of the metal, which is
obviously necessary for the action on sulfhydryl bodies proper.

## 2. Cobalt.

The reaction with cobalt is more complicated.  Disregarding
the so called cobaltiacs, which contain coordinatively bound $NH_3$
molecules, we find only one reference[1] in the literature of a cobalt
complex with a sulfhydryl body.  Rosenheim and Davidsohn (3)
obtained a dark brown compound by treating thioglycolic acid
with cobalt carbonate and attribute a definite formula to this
compound.  They consider it as an acid cobaltous salt of a cobal-
tous-thioglycolic acid complex.  This formula, however, does not
account for a fact which was unknown to these authors; to wit,
that the production of this colored complex takes place under
absorption of oxygen from the air.  A renewed investigation would
be worth while.  All observations now to be described hold just
the same for cysteine as for thioglycolic acid; whereas cysteine

[1] We disregard also the reaction of cobalt with $CH_3 \cdot COSH$ described by
Danziger (4) which is obviously quite different from that with compounds
of the type $SH \cdot CH_2 \cdot R \cdot COOH$, where the radical R may or may not contain
an amino group.

esterified at the SH group shows no color reaction with any metal salt at all.

A solution of cysteine at pH 7.5 to 8.5 in phosphate or borate gives with cobalt nitrate or sulfate a dark yellow-brown color which in high dilution has just a shade of olive. The color, however, arises only when oxygen is dissolved in the solution. If the reagents are freed from oxygen by a stream of nitrogen before being mixed, no color can be seen in an ordinary test-tube experiment with low concentrations of the reagents. The cobalt, which is precipitated by the anions of the alkaline buffer as an amorphous, faintly pink compound, is dissolved by cysteine to a complex which in the absence of oxygen is practically colorless. When this solution is shaken with air, the brown color just described arises and persists permanently. The first trace of oxygen led into the colorless solution produces a slightly more olive-green color which soon becomes browner. When the full, brown color is developed and the solution is diluted, the color will never completely match the olive-brown mentioned, but even in high dilution appears yellow-brown with scarcely a shade of olive. Subsequent deaeration for any length of time by a nitrogen stream does not destroy the color. When for a given amount of cobalt the amount of cysteine is varied, the maximum color of the oxidized complex is obtained when the ratio of cobalt atoms to cysteine molecules is about 1:3. Further increase of cysteine does not increase the color.

When the experiment is performed in larger bottles and a thorough deaeration is performed with highly purified nitrogen, as described in Paper I of this series (5), it can be seen that the soluble cobaltous cysteine is not quite colorless but has a very definite color too. When the amount of dissolved cobalt atoms to cysteine molecules is of the same order of magnitude (1:1 to 1:3), the color is a pure olive-green. When there is a great excess of cysteine, the color is a pure, delicate pink. According to the ratio of cysteine to cobalt, therefore, at least two different complexes may be formed combining cobalt and cysteine in different proportions.

The tendency of the cobaltous complex to be oxidized to the stronger colored brown complex is exceedingly strong. Besides molecular oxygen, ferricyanide and even organic dyestuff can work as oxidants in the absence of air. The dye is reduced to the leucodye, and the color of the oxidized brown cobalt complex arises.

The cobaltous complex of cysteine is not only one of the most powerful absorbents for oxygen gas, but also one of the most powerful reductants. It reduces instantaneously the dyes of the indophenol series, methylene blue, all of the indigosulfonates, and even phenosafranine. In correspondence herewith the cobaltous cysteine complex establishes a very strong negative potential at the platinum, gold, or mercury electrode which at pH 7.5, almost within the limits of error, equals the potential of a hydrogen electrode of the same pH. At pH 8 to 9, in borate buffer, the absolute value of the potential is the same as at pH 7.5 within the limits of error, hence the difference of a hydrogen electrode of the same pH and the cobalt-cysteine is greater. At pH between 6 and 7 the absolute value of the potential is less negative, not only absolutely, but also the difference between the potential of the hydrogen electrode of the same pH and the cobalt-cysteine electrode is greater than at pH 7.5. Taking the hydrogen electrode of the same pH as it is in the cobalt-cysteine system, as reference point, we may say that the reducing faculty of cobalt-cysteine is highest at pH about 7.8. Here this reducing faculty equals that of hydrogen of 1 atmosphere pressure. In any other range of pH the reducing faculty is smaller than the one of hydrogen of 1 atmosphere pressure. The optimum pH of the reducing faculty coincides with the optimum pH of color formation on mixing cysteine with cobalt.

A brown-colored complex can be obtained from the colorless cobaltous complex in the absence of oxygen also by cystine. One may think of cystine primarily being reduced to cysteine by the strongly reducing cobaltous cysteine complex. Thus, even cystine is an oxidant for the cobaltous complex. The color obtained by cystine is of the same shade as that obtained by the other oxidants, but less intense. The reaction is possibly not quite identical with the one obtained by other oxidants, which will be even more obvious in the measurement of potentials which are presently to be described.

Micro gas analysis in Warburg's apparatus was used to study the stoichiometry of the cobalt complex. Cobalt is no catalyst in contrast to iron. When present in minute amounts, such as would be suitable to show the catalytic effect in the case of iron, no oxygen consumption can be detected on mixing cysteine with a cobalt salt. When cobalt is present in greater amounts, oxygen con-

sumption takes place by the formation of the brown complex. With cobalt in excess, the amount of oxygen consumed depends only on the amount of cysteine and is two-thirds of that amount of oxygen which would be required to oxidize cysteine to cystine. With cysteine in excess, the amount of oxygen consumed depends only on the amount of cobalt and is the double of what would be necessary to oxidize the cobaltous state to the cobaltic.   With a great excess of cysteine, it cannot be entirely avoided that after the end of the rapidly proceeding oxygen consumption a further slow catalytic oxidation of the excess of cysteine by traces of iron may take place which may sometimes interfere a little with a really accurate estimation of oxygen consumption by the cobalt complex. The total oxygen consumption in a mixture of a small amount of cobalt and a large amount of cysteine is proportional to the amount of cobalt, and is independent of the amount of cysteine.   The oxygen consumption goes on rapidly, and the time for the establishment of equilibrium in the manometer is scarcely more than the time required for the establishment of the new gas equilibrium in the apparatus.   When a small amount of an iron salt is mixed with a large amount of cysteine, the total amount of oxygen consumption depends only on the amount of cysteine, the iron determining only the velocity of oxygen consumption, as Warburg has shown. When cobalt and cysteine are mixed, the total amount of oxygen consumed is proportional to the amount of cobalt up to a definite maximum value of the cobalt.   When in a series of experiments the amount of cobalt was kept constant and the amount of cysteine was varied, the maximum amount of absorbed oxygen was observed when the ratio of cysteine molecules to cobalt atoms was approximately 3:1.   The accuracy of this statement will be discussed in the "Experimental" part.   At any event, it is decidedly greater than 2:1 and smaller than 4:1.   Further increase of cysteine did not increase the amount of consumed oxygen.   This amount of oxygen absorbed in optimum ratio of cobalt to cysteine was 1 atom· of oxygen to 1 atom of cobalt or to 3 molecules of cysteine. When the ratio of cobalt to cysteine is different from 1:3, the amount of consumed oxygen is always in proportion to that; when cobalt is in excess with respect to this ratio, the amount of oxygen consumed is 1 atom of oxygen to 3 molecules of cysteine.   When cysteine is in excess, the amount of oxygen consumed is 1 atom of

oxygen to 1 atom of cobalt. Thus we may state that the fully oxidized cobalt complex is most likely one definite compound containing 3 molecules of cysteine to 1 atom of cobalt and arises from the cobaltous complex by consumption of 1 oxygen atom for 1 atom of cobalt. The simplest formulation by writing the cobaltous complex $Co^{II}$ $(SR)_2$, and the cobaltic complex $Co^{III}$ $(SR)_3$, when RSH stands for cysteine, does not fit into this observation, because according to such a formulation the oxygen consumption would be only half the observed one.

In agreement with the amount of consumed oxygen is the amount of consumed oxidant, such as ferricyanide or phenol-indophenol. When a definite amount of cysteine, dissolved in oxygen-free phosphate buffer containing cobalt to an amount of at least 1 atom of cobalt to 3 molecules of cysteine, is titrated with ferricyanide, 2 molecules of ferricyanide are consumed for 3 molecules of cysteine to oxidize the cobaltous cysteine to such an extent that an end-point of the titration is marked by the sudden rise of the potential. The titration detects, however, something new which cannot be recognized by the gas analytical method. The potential of the pure, oxygen-free cobaltous cysteine is at pH 7.5 equal to the potential of the hydrogen electrode at the same pH, as mentioned above. During the titration with ferricyanide, the potential very gradually becomes more positive in a smooth flat curve. When 1 molecule of ferricyanide to 3 molecules of cysteine is added, a sudden rise of the potential takes place, indicating an end-point of oxidation. This rise, however, leads only to a second level of potential, which is still the potential of a very strong reductant. When 2 molecules of ferricyanide are used up, the potential jumps suddenly into the potential range of the ferro-cyanide-ferricyanide system. Thus it becomes obvious that the oxidation takes place in two distinctly definite steps, each characterized by a sudden rise of potential. The solution, before addition of the oxidant, is slightly olive-green, as mentioned before. During the titration the color becomes dark brown, and no turning of color can be detected when the first level of potential rises to the second. No color change, at least none detectable without spectroscopic analysis, takes place when the primary oxidation product is further oxidized.

When the oxidation of cobaltous cysteine is performed with

phenolindophenol instead of ferricyanide, the total amount of oxidant used is equivalent to that in the ferricyanide titration. 1 molecule of indophenol is equivalent to 2 molecules of ferricyanide. The potential shows, however, a different course. A smooth curve is obtained and no step formation in the middle of the curve can be recognized. The shape of the titration curve in this case also is different from that of the well known curves obtained on titration of reversible oxidation-reduction systems. It is likely that the difference between the curves with ferricyanide and with indophenol may be attributed to the fact that indophenol can only oxidize by giving off 2 hydrogen atoms (respectively electrons) at once, owing to the quinone structure of the dye. Even if cobaltous cysteine can be oxidized in two separate steps, both steps of oxidation will take place at once with an oxidant such as indophenol, whereas ferricyanide may be able to oxidize the two steps in succession.

As far as we can see, the case of cobaltous cysteine is the first one in which the difference in these two kinds of oxidants is manifested. We may speak of ferricyanide as a *single step oxidant*, of indophenol as a *double step oxidant*, and of cobaltous cysteine as a *two single step reductant*.

Cobaltous cysteine reacts, furthermore, with cystine, as mentioned above. The complex obtained in this reaction is, however, probably a little different from the other oxidized forms of the cobalt complex. As cystine, at room temperature, is exceedingly slightly soluble, and besides, in so far as it is soluble at all, exceedingly slowly soluble at the pH range of the phosphate buffers, the experiment is more easily performed in borate buffer. (About solubility of cystine at different pH see Sano (6).) When a borate buffer (pH 9.1 before addition of the other substances) is saturated with cystine by boiling, cooled down, mixed with cysteine, deaerated by nitrogen, and then cobalt is added, the olive-green color of the cobaltous cysteine appears only in the beginning and turns gradually more and more to brown. This, however, never became as dark as by oxidation with any of the other oxidants. (Cystine alone gives no color reaction with cobalt.) When the light brown solution, obtained by the action of cystine, was afterwards exposed to air it kept its light brown and never turned as dark brown as when directly oxidized by oxygen without cystine.

When the potential is measured in a vessel in which the green cobaltous complex is gradually acted upon by cystine, the potential is in the beginning almost as strongly negative as in the pure cobaltous complex and rises quite gradually. No sharp potential can be obtained in these solutions. When the action of the cystine becomes perceptible, the potential shifts very gradually to the positive side, without ever surpassing the first level of the potential as obtained in the first step of oxidation with ferricyanide, and after that it even returns on standing into the more negative range.

### 3. Iron.

At the acid reaction of a solution of cysteine hydrochloride in water or in 0.01 N HCl, ferric chloride produces a deep indigo-blue color as described by Baumann (7) and by Mathews and Walker (8). This fades away in a short time and cannot be restored by oxygen. It arises again, however, on subsequent addition of some $FeCl_3$ as long as there is unoxidized cysteine present. Ferric ions give a complex with cysteine which is blue at acid reaction, but is unstable in that spontaneously the ferric ion is reduced to the ferrous state and cystine arises. The ferrous state, at acid reaction, is not oxidized to the ferric state by molecular oxygen. There is scarcely any catalytic effect of iron at acid reaction, but the oxidation of cysteine is only equivalent to the amount of ferric ions available. The faded out solution stays colorless even after addition of alkali, provided no oxygen is present. This is in contrast to a statement by Lyons (9). His observations deal with thioglycolic acid but as will be shown it does not differ in this respect from cysteine, and Lyons' interpretation, if it be true at all, should also be applicable to the case of cysteine. Lyons observed the following. When the blue color of the ferric complex at acid reaction has faded out and the solution is made alkaline, a violet color arises. He interprets this observation thus: the blue ferric complex is reduced, at the expense of the formation of the disulfide, to a ferrous complex which is colorless at acid reaction but which is violet in its ionized state at alkaline reaction. The following experiment, however, shows that this interpretation is not adequate. When the blue color at acid reaction has faded out, and then the solution is carefully freed from air by bubbling with

nitrogen, addition of alkali (say borate buffer or ammonia) does not produce any color. The violet color arises only after the deaerated alkaline solution has been shaken with air. By this experiment it can be proved that the violet color at alkaline reaction is not simply the ionized form of a ferrous complex which is colorless at acid reaction, but that the violet compound is an oxidation product of the ferrous complex. This will become even clearer as we go now to the direct interreaction of cysteine and iron at alkaline reaction. In order to understand this reaction one has to start all experiments in a deaerated solution. On this condition the solution of cysteine of pH 7 to 8.5 gives no color reaction with a ferric or a ferrous salt, even under those conditions where the color of the relative faintly colored cobaltous complexes can easily be recognized. When the colorless and soluble ferrous cysteine complex is shaken with air, the violet color arises as described by Warburg and Sakuma (10). It fades away gradually, but can be obtained again by subsequent bubbling with air as long as there is unoxidized cysteine left  The violet complex must be considered, therefore, as an oxidized form of the ferrous complex analogous to the oxidized cobalt complex, but whereas the latter is stable the oxidized iron complex is spontaneously rearranged to the ferrous state and cystine is formed. The ferrous ion forms a complex with a new portion of cysteine. The ferrous complex of cysteine is directly oxidized by oxygen, and so a catalytic effect of iron is established as described and interpreted by Warburg. The violet color, with a given amount of cysteine, is strongest with a relatively small amount of iron. When the ratio of Fe and cystine is of the same order of magnitude, the violet color never becomes as dark as with smaller amounts of iron. Probably the fading of the color depends on the total amount of iron, and with too much iron the rate of fading is so high that the concentration of the colored compound at any instant is low. With relatively small amounts of iron and a large excess of cysteine, the color may be very deep and relatively stable. This is especially true with thioglycolic acid. Here a trace of iron with an excess of this acid at pH 8 to 10 gives a rather stable violet color which can be used as a sensitive reagent for iron, as shown by Lyons.

The ferrous complex and cobaltous complex behave alike in so far as both can be easily oxidized by molecular oxygen, but are

different in so far as in the case of cobalt the oxidization product is very stable and forms the end-product of the reaction, whereas in the case of iron the oxidation product is very labile and involves the catalytic effect of iron.

As regards the behavior towards iron, everything is alike with cysteine and thioglycolic acid except for the difference of the pH optimum for the oxidation catalysis with iron described by Dixon and Tunnicliffe (11).  When the colorless ferrous cysteine complex in absence of any trace of oxygen is oxidized by phenolindophenol, the same purple-violet transient color arises simultaneously with the reduction of the blue dye.  It fades out gradually.  When, however, the colorless ferrous cysteine complex is treated with cystine in the same way as described above for cobalt, no color arises.

A chemical system may be built up by the following constituents: cysteine, metal-ion, oxidant.  Then, the most stable form of all kinds of molecules containing cysteine which can be formed from these constituents is the oxidized cobalt-cysteine complex when the metal is cobalt; it is free cystine when the metal is iron. That compound, which is intermediary, unstable, and unaccessible therefore for a chemical analysis in the case of iron, is stable and suitable for chemical analysis in the case of cobalt.  This fact makes the cobalt complex valuable for the understanding of the oxidized labile iron complex and the chemistry of the iron catalysis.

The reduction potential of ferrous cysteine was not investigated in detail for the following reason.  When a mixture of ferrous salt and cysteine is made in the buffer, there will be generally an excess of either cysteine or iron, unless the ratio of iron and cysteine is just the one corresponding to the complex, and even in this special case it is doubtful whether the affinity of the constituents is high enough to bring about a complete complex formation or only an equilibrium of the complex with its components.  In the case of cobalt the difficulty could be overcome by adding an excess of cobalt salt. This is not feasible with iron.  For iron salts in an alkaline or even neutral medium, *e.g.* in phosphate buffer pH 6.8 (*i.e.* ferrous hydroxide or basic ferrous salts) are strong reductants themselves, consume oxygen, reduce dyestuffs, and develop a strong reducing potential at the electrode.[2]  With an excess of iron, it cannot be

[2] We are indebted to Dr. V. K. La Mer for calling our attention to the fact that a ferrous salt in an alkaline medium reduces methylene blue at

decided how much the presence of this excess influences the potential of the ferrous cysteine.   On the other hand, with an excess of cysteine, it cannot be decided how much the presence of free cysteine interferes with the effect of ferrous cysteine upon the electrode. As the mechanism of the establishment of these potentials is not the one of reversible systems and it is not sure at all whether these potentials are due to a thermodynamically well defined state of equilibrium throughout the whole system, the problem had to be postponed until some time later.   It may be said, however, even now that the potentials obtainable *in maximo* with ferrous cysteine are not quite as negative as with cobalt-cysteine and are even less negative than the potentials described for pure cysteine in Paper I of this series (5).   It had been mentioned in that paper that addition of small amounts of iron salts to cysteine does not appreciably affect the potential of cysteine at a platinum, gold, or mercury electrode.   This statement was made for very small amounts of iron salts.   Addition of iron in an amount comparable to that of the cysteine was beyond the scope of that earlier paper and does appreciably affect the cysteine potential.

<div align="center">EXPERIMENTAL.</div>

<div align="center">1. *Oxygen Consumption by Cobaltous Cysteine.*</div>

The oxygen consumption was measured in Warburg's micro respiration apparatus with Barcroft manometers.   The vessel had a side arm which contained cysteine hydrochloride, purified with acetone according to Warburg, practically iron-free, dissolved in a volume of 0.10 cc.   The main vessel contained phosphate buffer, pH 7.73, and cobalt in form of cobalt sulfate, in a volume of 2.00 cc. together.   The cobalt was precipitated as a slightly pink cobalt phosphate.   After establishment of temperature equilibrium the cysteine and the buffered cobalt were mixed.   The olive-brown color of the complex developed as oxygen was consumed.   It will be shown that the total oxygen absorption increases with increasing ratio of cysteine to cobalt up to a definite

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room temperature and establishes, in the absence of oxygen, a strong reduction potential.   The oxygen consumption of ferrous compounds in an alkaline medium has been known for a long time.

maximum ratio of approximately 3:1.    It is easy to determine very accurately the amount of oxygen consumed when this ratio is smaller; *i.e.*, when cobalt is in excess with respect to this optimum ratio.    The oxygen consumption is finished in a short time and then the level of the manometer keeps constant for any length of time (say 6 hours of observation).    The following is an experiment of this type.

0.02 millimols of cysteine + 0.01 milliatoms of cobalt consumed (*a*) 66.2 cmm., (*b*) 65.0 cmm. of oxygen gas of standard condition, or
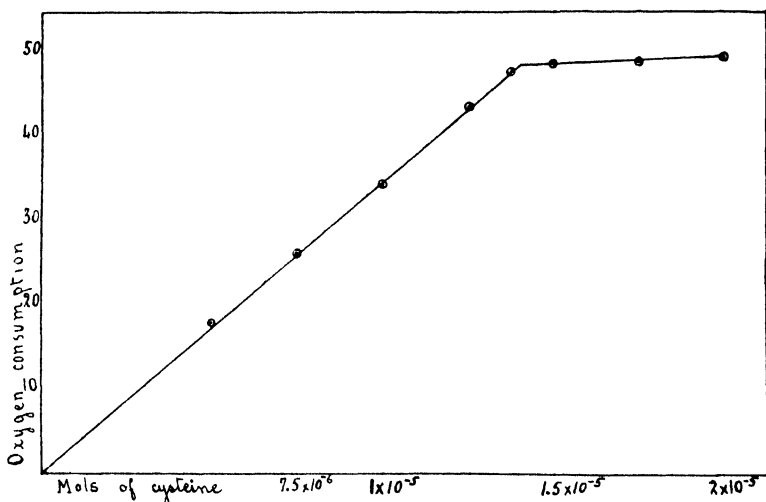


Fig. 1. Oxygen consumption of cobaltous cysteine in cmm. $5 \times 10^{-6}$ gram-atoms of cobalt were mixed with varied amounts of gram-molecules of cysteine.

0.0296 respectively 0.0290 millimols of $O_2$.    The same amount of this cysteine preparation, catalytically oxidized with a trace of iron salt, consumed 102.0 cmm. or 0.0455 millimols of $O_2$ (calculated for the same mass of theoretically pure cysteine hydrochloride: 0.0500 millimols of $O_2$).    The amount of oxygen, therefore, consumed with an excess of cobalt, is $\dfrac{0.0290}{0.0455} = 0.64$ or practically two-thirds of that amount of $O_2$ consumed by the same amount of the same cysteine preparation in the iron catalysis. .

In a series of experiments (Fig. 1) the amount of cobalt was kept

constant, $5.00 \times 10^{-6}$ milliatoms of Co, and varied amounts of cysteine were used as indicated in the abscissa. The oxygen consumption is proportional to the amount of cysteine up to a definite amount. The optimum amount of cysteine is reached with 1.4 or $1.45 \times 10^{-5}$ mols of cysteine, *i.e.* cysteine:cobalt $= 2.8:1$. The difficulty of getting this ratio with complete accuracy is due to the fact that the amount of cysteine, which is in excess of the amount combined with cobalt, is gradually catalytically oxidized, obviously by unavoidable traces of iron in the cobalt preparation. The absorption of oxygen by the cobalt complex is very rapid, and the catalysis of the excess of cysteine very slow. This fact permits the reading of the end-point of the oxygen consumption due to the cobalt complex with a close approximation indeed, but not with the utmost accuracy. For this reason we may consider the ratio of cysteine molecules to cobalt atoms in the oxidized complex $= 3:1$ within the present limits of error of these experiments. In any case, this ratio is decidedly greater than $2:1$ and smaller than $4:1$.

### 2. Titration of Cobaltous Cysteine with Potassium Ferricyanide.

The results of this experiment are shown in Fig. 2. Bright platinum was used as electrode. The potential of the normal hydrogen electrode has been taken as zero. The total volume in the beginning was 40 cc. Sörensen's phosphates in the following ratio were used. Secondary phosphate:primary phosphate $= 9:1$. The amount of cysteine hydrochloride $= 1.5 \times 10^{-4}$ gram-molecules. The amount of cobalt (as $CoSO_4 \cdot 7H_2O$) $= 1 \times 10^{-4}$ gram-atoms. This is an excess of cobalt with respect to the one combined with cysteine in the oxidized form of the complex, this combined amount of cobalt being $0.5 \times 10^{-4}$ gram-atoms. The concentration of $K_3Fe(CN)_6 = 0.1$ M. The temperature $= 23°$.

The dry cysteine hydrochloride is kept in the electrode vessel in a spoon above the liquid to prevent oxidation, and added to the solution only after 1 hour of bubbling purified nitrogen through the titration vessel.

The potential of cobaltous cysteine is established in the course of $1\frac{1}{2}$ to 2 hours after the addition of cysteine. With the buffer employed the final value is practically equal to the hydrogen potential of the solution, as calculated from the buffer and as checked with a freshly platinized platinum electrode in hydrogen gas, at
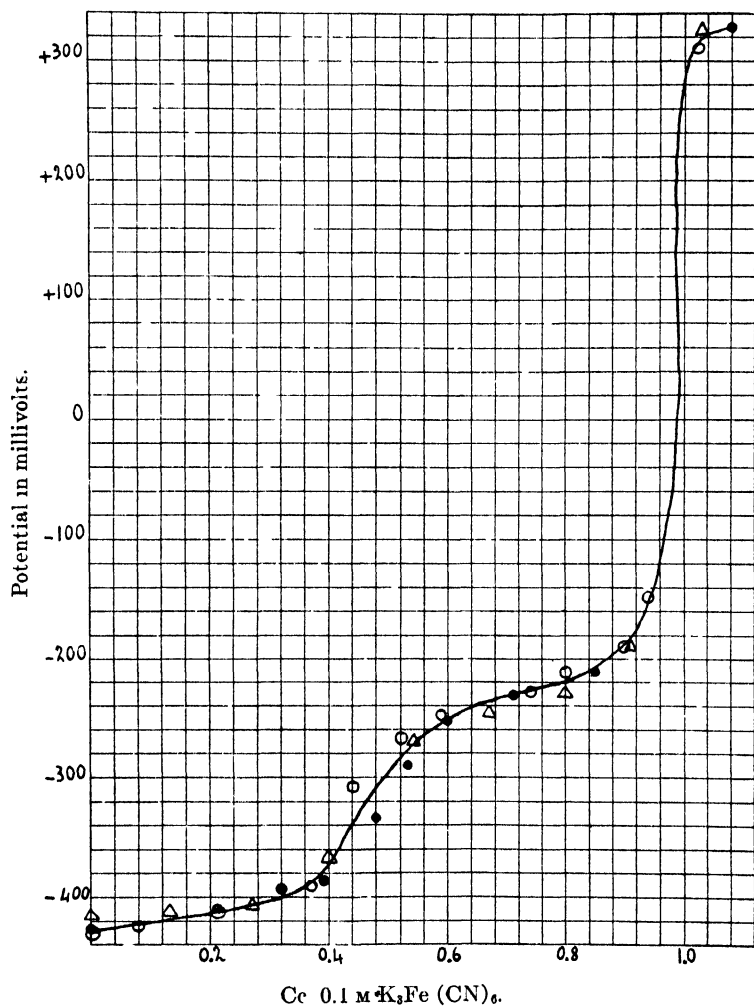
FIG. 2 Titration of cobaltous cysteine with potassium ferricyanide. The abscissæ represent cc. of ferricyanide; the ordinates, potential in millivolts, referred to the normal hydrogen electrode. Blank platinum electrodes: ●, △, and ○ refer to three separate experiments.
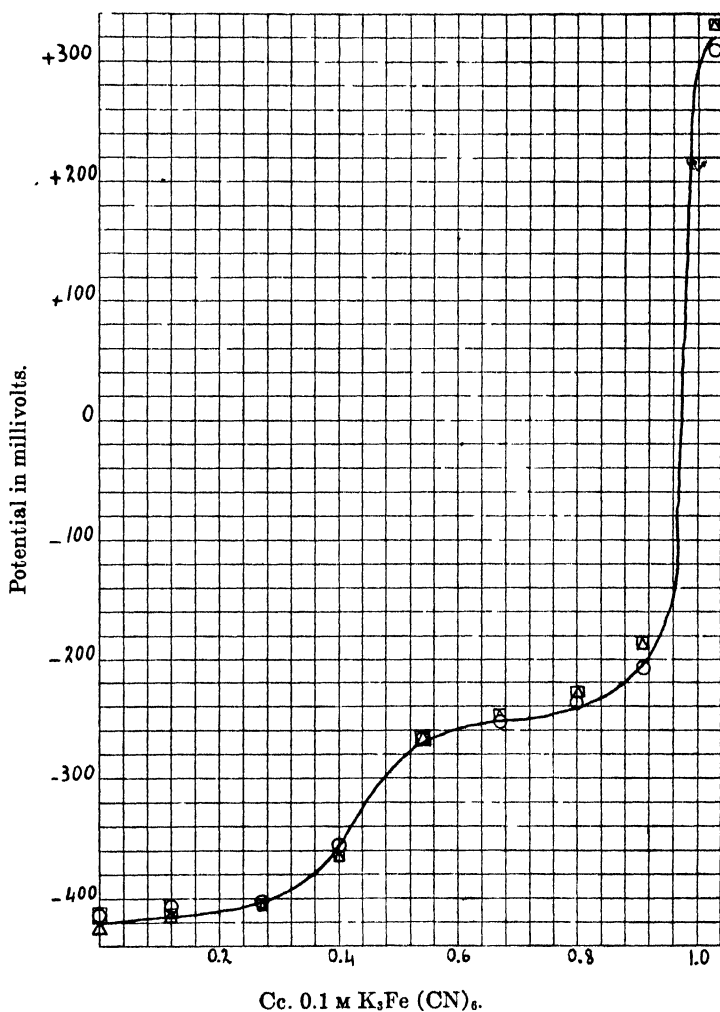
Fig. 3. Titration of cobaltous cysteine with potassium ferricyanide, with various electrodes. The abscissæ and ordinates have the same significance as in Fig. 2.   □ represents readings with gold-plated electrode; ○, bright platinum electrode; △, mercury electrode.

the end of the titration.    (In borate buffer the potential of cobaltous cysteine in $N_2$ at blank platinum is about 100 millivolts less negative than the potential of a platinized platinum electrode in hydrogen.)    The plotted curve represents three different titration experiments, indicated by three different marks.
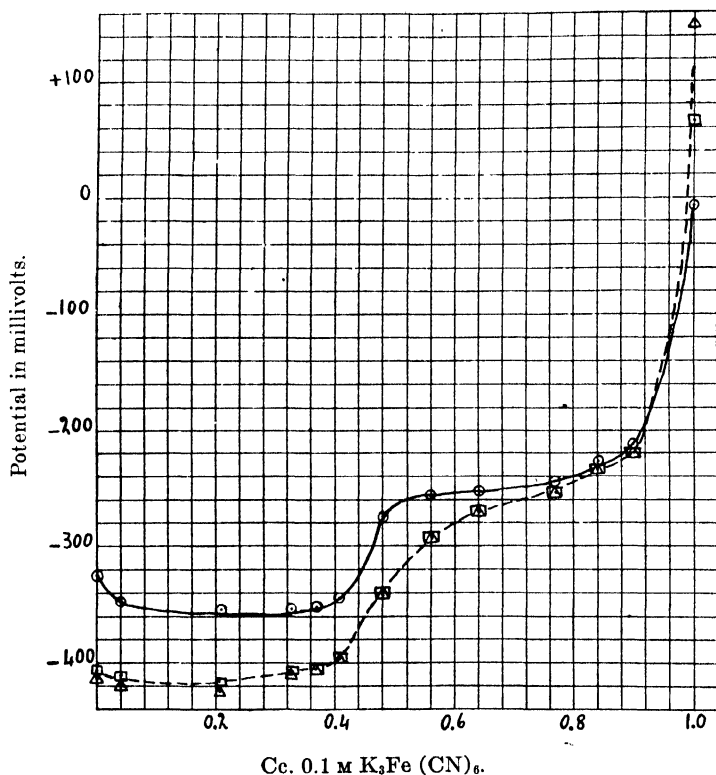


Fɪɢ. 4. Titration of cobaltous cysteine with potassium ferricyanide. O represents results with the mercury electrode; □, gold-plated platinum electrode; △, bright platinum electrode.

·    The establishment of the potential after each addition of ferricyanide takes some time and can be largely shortened when the vessel is thoroughly shaken from time to time.    The stirring effect produced by the bubbling of the gas is not sufficient to obtain the definite value within reasonable time.

In the first part of the curve (Fig. 2), the potential drifts to the positive side on addition of ferricyanide and returns gradually to the negative side so as to reach a definite value within 10 to 20 minutes. In the next part, where the first level rises into the second, the potential is less well reproducible and more easily polarized. In the following part, representing the second level of the potential, the establishment of the potential takes place by a gradual drift from a more negative value to the definite one in contrast to the behavior of the electrode in the first part of the titration. The potentials are well defined and not easily polarized in this second level.

The total amount of oxidant used is, again, two-thirds of what would be necessary to oxidize all of the cysteine to cystine.

The color of the liquid is grass-green immediately after addition of cysteine to the cobalt-containing buffer. The first drops of ferricyanide turn it to brown. No change in color can be detected while the first level of the potential is rising into the second.

The buffer used consisted of 36 cc. of $N/15$ secondary phosphate (Sörensen) and 4 cc. of primary phosphate. By taking into consideration the addition of cysteine hydrochloride and cobalt sulfate, the pH of such a mixture may be roughly evaluated to 7.3 or 7.4. In fact, a pH measurement in the liquid after the end of the titration experiment, performed with a freshly platinized electrode in hydrogen gas, showed pH 7.4, or in other words a potential of 0.430 volt. This is only about 5 to 10 millivolts more negative than the potential of the cobaltous cysteine in $N_2$ against blank platinum or gold.

*Titration of Cobaltous Cysteine with Potassium Ferricyanide, with Various Electrodes.*

The results of this experiment are shown in Fig. 3. The concentrations of the solutions are the same as in the experiments shown in Fig. 1.

It is worth while mentioning that during the second step of oxidation (from 0.5 cc. of ferricyanide on) the potentials at the mercury electrode reach equilibrium almost instantaneously, while those at gold-plated platinum and bright platinum take from 25 to 30 minutes.

*Titration of Cobaltous Cysteine with Potassium Ferricyanide.*

The results of this experiment are shown in Fig. 4.   The concentration of the solutions was the same as in Fig. 2.



Cc of M/30 phenolindophenol
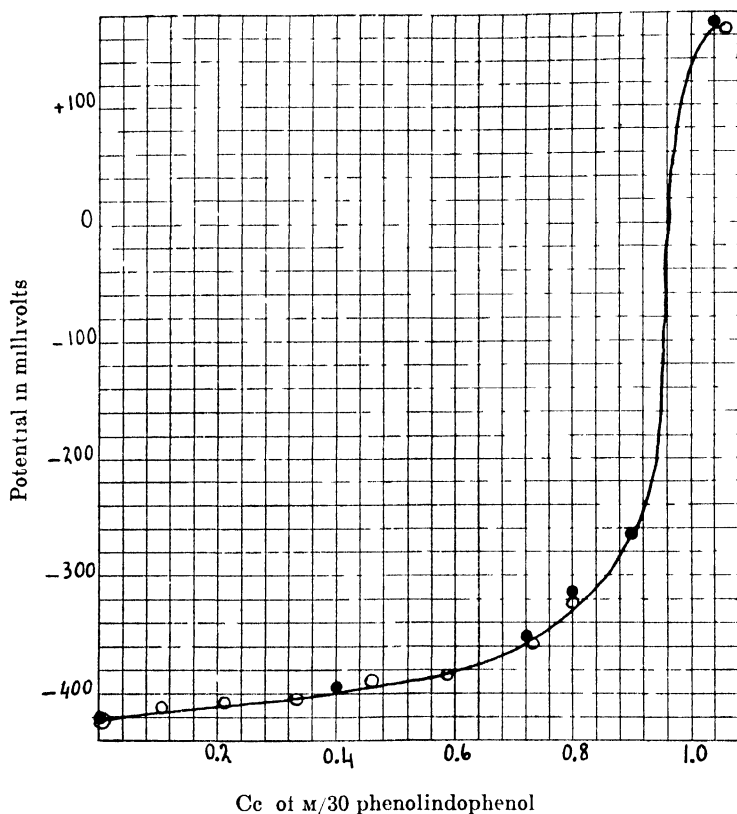
FIG 5  Titration of cobaltous cysteine with phenolindophenol     Blank platinum electrode     ● and ○ refer to separate experiments

In this case the establishment of the potential before the addition of ferricyanide was exceedingly sluggish.   Therefore, we decided to begin the titration before the definite potential was reached. After the first addition of oxidation, however, the potential went to the negative side instead of the positive, and from there the potential reached a definite value within reasonable time, which

was even a little more negative than in the earlier cases, for bright and gold-plated platinum, but less negative for mercury. The potential curve during the first level of titration is even flatter than in the other experiments. Despite these deviations the appearance of the two steps of oxidation is quite obviously the same as before.

### 3. Titration of Cobaltous Cysteine with Phenolindophenol.

Fig. 5 shows the results of this experiment. The electrode employed was bright platinum.

The buffer used was Sörensen's phosphate $M/15$, pH 7.73.

The amount of cysteine $= 1 \times 10^{-4}$ gram-molecules.

The amount of cobalt (as $CoSO_4 \cdot 7H_2O$) $= 3.38 \times 10^{-5}$ gram-atoms.

The concentration of phenolindophenol[3] (in phosphate buffer) was $M/30$. Two different titrations are plotted.

The amount of oxidant totally used is equivalent to that of ferricyanide, referred to the cysteine. Cobalt was present, again, in slight excess with respect to the amount necessary to form the complex. The curve of titration shows nothing of those two levels as obtained with ferricyanide.

#### SUMMARY.

1. Nickel gives at pH 7 to 8 a Bordeaux red complex with cysteine in absence or in presence of oxygen.

2. Cobalt gives at pH 7 to 8 with cysteine in absence of oxygen a cobaltous complex, which is usually slightly olive-green, but pink when there is a large excess of cysteine.

3. The cobaltous complex is rapidly oxidized by air, organic dye-stuffs, or ferricyanide to a brown complex. The ratio of cobalt to cysteine in this oxidized complex is 1:3.

4. The amount of oxygen consumed to oxidize a mixture of 1 atom of cobalt and 3 molecules of cysteine to the brown, stable complex is 0.5 molecule of $O_2$.

5. The amount of ferricyanide or of phenolindophenol reduced by cobaltous cysteine is equivalent to the oxygen consumption.

[3] We are indebted for this very pure sample of phenolindophenol to the I. G. Aktiengesellschaft Farbenindustrie, Leverkusen, Germany.

6. When the titration of cobaltous cysteine is performed with ferricyanide, the course of the potential shows two distinct steps of oxidation.

7. The potential of pure cobaltous cysteine at pH 7.5 practically matches that of the hydrogen electrode for the same pH. Cobaltous cysteine is one of the most powerful reductants at pH 7.5 to 8.

8. Cobaltous cysteine, in absence of oxygen, gives a brown complex with cystine.

9. Cobalt and nickel are no catalysts for the oxidation of cysteine.

10. The violet iron complex of cysteine at pH 7 to 8 corresponds to the oxidized form of the cobalt complex, as far as one can tell, but is different in so far as it is labile. This fact leads to the formation of cystine and makes iron a catalyst.

11. The study of the cobalt complex is important because the end-product of its oxidation is a compound which is analogous to an intermediary compound in the case of iron, and will permit the study of the intermediary state in the iron catalysis.

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# THE CARPENTER FORM OF THE HALDANE GAS ANALYSIS APPARATUS.

## CHANGES MADE IN THE APPARATUS AND DETAILS REGARDING ITS USE.

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The apparatus devised in this laboratory (Carpenter, 1923) for the analysis of chamber air has now been in use several years and has given satisfaction in the investigations carried on in cooperation with the Institute of Animal Nutrition at the University of New Hampshire (Benedict and Ritzman, 1927). Its use in a European laboratory has recently been described by Grafe, Strieck, and Otto-Martiensen (1927) whose experience with it substantiates the claims made for the apparatus in the original description. A modified form of this apparatus has been described by du Vigneaud (1927).

An important modification of the potassium pyrogallate pipette in the Haldane apparatus has been made by Professor J. C. Bock[1] of the Department of Pharmacology, University of Copenhagen, who has placed a glass bead in a bulb of the pipette in such a way that it acts as a one-way valve. The pipette was further modified so that the solution would return through side tubes and thus make fresh solution available for the absorption of oxygen. The description of a pipette on the same principle has been given by Strieck (1928). This form of potassium pyrogallate pipette has been adapted in this laboratory to the Haldane-Carpenter apparatus, which has been changed in some details. A description of the apparatus in its present form and of a modification of the combustion apparatus for methane, and further details regarding the use of the apparatus are given in the following pages.

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[1] Personal communication from Professor August Krogh to Professor Francis G. Benedict.
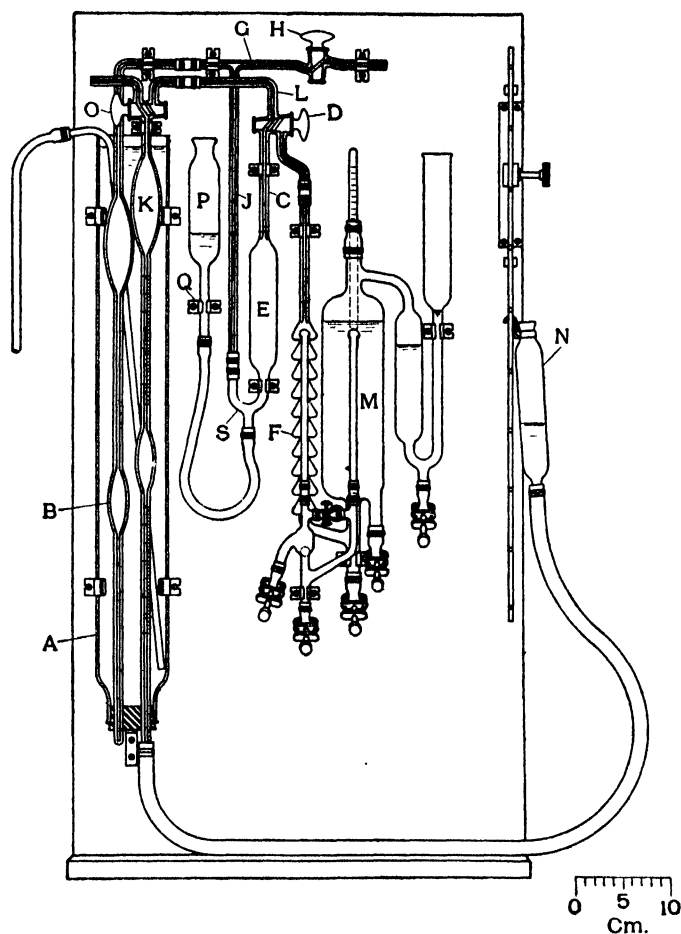
FIG. 1. The Carpenter form of Haldane gas analysis apparatus. The
apparatus consists of the burette, $K$, compensator tube, $B$, water jacket, $A$,
potassium hydroxide pipette, $E$, with its levelling bulb, $P$, the modified
form of potassium pyrogallate pipette, $FM$, and the mercury levelling bulb,
$N$, connected to the burette, $K$.

*Changes in Apparatus.*

A diagrammatic sketch of the apparatus in its present form for determination of carbon dioxide and oxygen is shown in Fig. 1. The following changes are a portion of the improvements which have been made: The water jacket, $A$, is tapered and flared slightly at the bottom so as to permit the use of a smaller rubber stopper. The stopper fits tightly and makes a water-tight closure. The lower end of the compensator tube, $B$, is closed by fusing instead of being closed with a rubber tube and pinch-cock. The latter arrangement has caused much trouble, because it was difficult to remove air bubbles from the connection when the tube was partly filled with water. The capillary tube, $C$, leading from the three-way stop-cock, $D$, to the potassium hydroxide pipette, $E$, has been shortened and straightened and the curved capillary has been placed on the side leading to the pipette, $F$, which holds the potassium pyrogallate.

When the apparatus is set up, the capillary tee, $G$, and stop-cock $H$, and the compensator, $B$, are adjusted at such a height that the volume included between the level of the potassium hydroxide in the capillary, $J$, and the point which the top of the water in the jacket, $A$, touches on the compensator, $B$, just equals the volume from the water level on the burette, $K$, to the level of the potassium hydroxide in the capillary, $C$, of the pipette, $E$.

The volumes of these portions of the apparatus are determined by filling the parts with mercury, weighing the mercury, and calculating the equivalent volume from the density of the mercury at the temperature at which the determination is made. In practice, a mark is made on the burette, $K$, at the level of the water in the water jacket. The capillary and stop-cock of the burette and angle above the stop-cock are then filled with mercury, the weight of which is determined. Similarly, the capillaries, $L$ and $C$, of the potassium hydroxide pipette and stop-cock $D$ are filled with mercury to the point which the level of the potassium hydroxide touches and the mercury is weighed. The sum of the two volumes computed from these weighings gives the volume to which the part of the compensator, $B$, outside the water jacket and the capillary tee to the surface of the potassium hydroxide in $J$ must be adjusted. The volumes of the latter capillaries are determined in much the same manner as above. From the volume in a given length of one

of the capillaries, the height to which the compensator, $B$, and capillary tee, $G$, have to be set is estimated, and a determination of the corresponding volume is made by weighing the mercury required to fill the capillaries to the estimated points. In order to make adequate compensation for the curves in the capillaries between the burette, $K$, and the potassium hydroxide pipette, $E$, the capillary leading to the stop-cock, $H$, which opens to the outside air, has been lengthened. When this compensation in the volumes of the capillaries outside the water jacket is exactly made, an air sample can be analyzed with a change of 1° in the external temperature during the half hour required, without appreciable error in the results. In other words, the apparatus compensates for changes in the environmental temperature.

*Modified Pipette for Potassium Pyrogallate.*—The most fundamental change in the apparatus is the new pipette, $FM$, for potassium pyrogallate. A diagrammatic sketch of the pipette alone is shown in Fig. 2, the main portion being the same as in the earlier form of the apparatus except that the reservoir bulb, $E$, is much larger. The modification is as follows: At $Q$ on the uppermost bulb, $A$, of the series of ten conical bulbs, a connecting tube, $B$, is fused which projects outward at a slight angle and extends downwards to the point $C$ where it is shown as open at the end. A tube, $D$, similar in construction, is fused into the upper part of the large bulb, $E$, and extends downwards to $L$. The separated portion, $FGHJ$, is a device of glass which is attached at $F$ and $G$ to the two side tubes, $B$ and $D$, by means of rubber tubing when the apparatus is in use. At $M$, where the tube, $P$, opens downward from the bottom of the large bulb, $E$, is a steel ball 9.5 mm. in diameter (standard $\frac{3}{8}$ inch ball bearing ball) which fits snugly into the tube, $P$. The tube has been ground at that point and the side opening, $N$, is large enough for the steel ball to be rolled into place from the outside. In the part, $FGHJ$, is also a steel ball at $K$ which fits snugly into the tube, $J$, which has been ground at $K$, and at $H$ is an opening large enough for rolling the ball in or out. At $R$ and $S$ are openings which are connected with rubber tubing. This rubber connection can be closed by a spring pinch-cock. The open tee at $U$ is used for the insertion of a thermometer or for filling the pipette with the pyrogallate solution.

After the part, $FGHJ$, is attached to the main portion of the
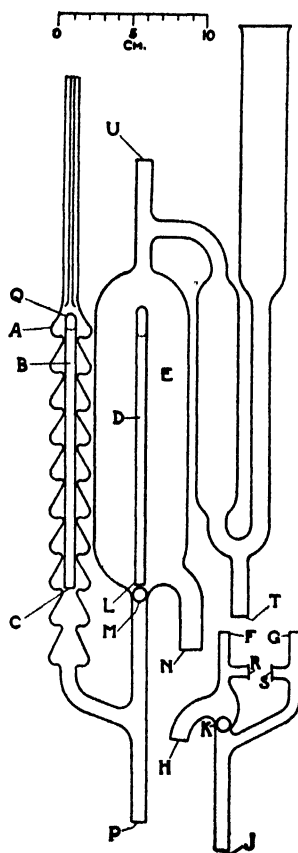
FIG. 2. Detail of modified potassium pyrogallate pipette. The apparatus consists of a series of bulbs beginning at $A$, and a large reservoir, $E$, both of which have side tubes, $B$ and $D$. The large bulb, $E$, is sealed at the bottom by the movable steel ball, $M$. The part, $FGHJ$, (shown detached) is connected at $F$ and $G$ to C and $L$ by rubber tubing. This part is also sealed by the steel ball, $K$. When the pipette is in use the pryogallate passes downwards from $A$ and upwards into $E$, and reversely out through $D$, past $K$, and upwards through $B$, and back to $A$. The opening, $U$, at the top, is for a thermometer and for filling the pipette with the pyrogallate solution. At $P$, $J$, and $T$ are openings for removing and replacing liquids. $H$ and $N$ are openings through which the steel balls, $K$ and $M$, are rolled into place.

pipette, short pieces of rubber tubing are wired on at $N$, $H$, and $J$ and on these are placed screw pinch-cocks. Pieces of rubber tubing and screw pinch-cocks are also attached to $P$ and $T$. When all parts have been connected, the pipette is filled with potassium pyrogallate solution through $U$ until the ten bulbs are full and the large bulb is filled until the liquid is above the side tube, $D$. The spring pinch-cock on the connection from $R$ to $S$ is then closed and the apparatus is ready for use.

The analysis is carried out in the usual way so far as burette reading and absorption of carbon dioxide are concerned (see Fig. 1). When the oxygen is to be removed from the gas, stop-cock $D$ is opened and the sample is forced down the capillary tube into the series of bulbs, beginning at $A$ (see Fig. 2). As the steel ball fits tightly at $K$, practically none of the gas passes into the side tube, $B$, and consequently the steel ball at $M$ is raised and the level of the potassium pyrogallate rises in the large bulb, $E$. When the mercury reservoir attached to the burette of the apparatus is lowered, the steel ball at $M$ seats, and prevents the solution from passing downward toward $P$. The solution therefore flows from the top of the large bulb, $E$, into the side tube, $D$, and passing downwards through $D$, it lifts the steel ball, $K$, and flows through the side tube, $B$, into the series of bulbs at the point $Q$, thus flushing and rinsing the bulbs while filling them. When all the gas has been drawn from the series of ten bulbs, they are filled with the potassium pyrogallate solution which has flowed into them through the tubes, $D$ and $B$. There is thus a circulation of the potassium pyrogallate solution in the operation of removing the oxygen from the gas. The advantages of the modified pipette are that fresh unused solution is brought into the series of bulbs and at the same time they are rinsed so that more solution is exposed to the action of the gas, and that the bulb, $E$, is much larger than the one in the earlier form of the pipette and so the solution may be renewed less frequently. With the earlier form of reservoir, the solution had to be renewed after every ten analyses, but with this larger bulb, 75 analyses may be carried out without renewal of the pyrogallate solution.

The routine for absorption of oxygen is to pass the sample into the potassium pyrogallate pipette ten times, then twice into the potassium hydroxide pipette, five times again into the potassium pyrogallate and twice into the potash solution, five times into the

potassium pyrogallate, and three times into the potash solution, and then to set the levels and read the burette. To confirm complete absorption of the oxygen, pass the gas into the potassium pyrogallate pipette five times, and three times into the potash solution, and read. During absorption the spring pinch-cock on the rubber tube between $R$ and $S$ is closed, but when the level of the potassium pyrogallate is set for the reading of the burette, the *cock should be open,* because when the pinch-cock is closed, an exact setting of the level of the potassium pyrogallate cannot be made.

*Modification of Combustion Unit for Methane.*—A modification of the gas analysis apparatus for chamber air has been described (Carpenter and Fox, 1926) as designed for determining combustible gas or methane in the air of respiration chambers. The glass tubes into which the platinum wire was fused in this arrangement were inside the combustion pipette, and when combustions were carried out, the tubes carrying the wires occasionally cracked because they were surrounded with cold mercury before they were adequately cooled. In order to prevent this, a form of combustion pipette constructed (Fig. 3) according to Weaver and Ledig (1920) has been adapted to the apparatus. The advantage of this form is that it may be made up as a separate unit and easily replaced in the apparatus in case the combustion wire is melted. At no time does liquid come in contact with the combustion wire. The unit contains 25 cm. of No. 33 Brown and Sharpe gauge platinum wire, 0.18 mm. in diameter, and is connected in series with an external resistance of 40 to 42 ohms. A current of 2.3 to 2.5 amperes from a 110 volt source is used to heat the wire.

When it is known that methane is the only combustible gas in the air, the determination can be shortened by inserting the combustion unit into the gas analysis apparatus between the burette and the potassium hydroxide pipette. The procedure in the analysis is to remove first the carbon dioxide in the air sample and then to burn the gas. The result of the arrangement is that, as the gas is passed backward and forward over the heated wire and into and out of the potassium hydroxide pipette, the carbon dioxide is absorbed as fast as it is produced in combustion. Thus the operations of combustion and absorption are carried on at the same time and the procedure is simplified for the determination of methane

in chamber air.　　As the wire, glass tubing, and contents are heated considerably during the combustion, it is necessary to surround
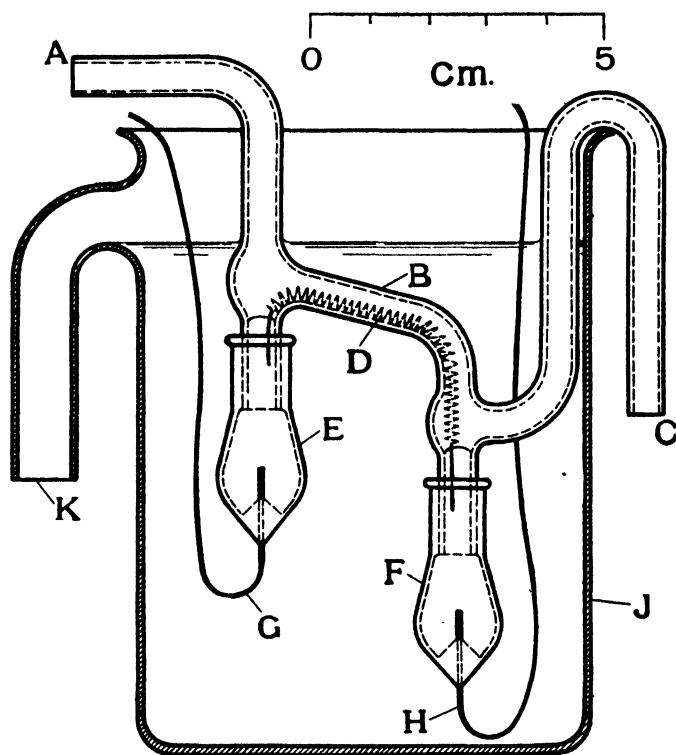


FIG. 3. The Weaver and Ledig form of combustion pipette.　The unit is inserted in the Haldane-Carpenter apparatus, connection being made at $A$ and $C$ to the measuring burette and the potassium hydroxide pipette. Wires $G$ and $H$ are sealed into the glass cups, $E$ and $F$, which are fastened to the tube, $ABC$, by Khotinsky cement.　The combustion wire, $D$, is wound in the form of a spiral and dips into mercury in the cups, $E$ and $F$. The beaker, $J$, contains water surrounding the combustion unit, and over-flow through $K$ provides for the running off of the water.

this portion with water at room temperature.　A side outlet is provided in the upper portion of the beaker containing the water so that excess water may flow out into a receptacle.　A container suspended above the gas analysis apparatus furnishes the supply

for changing the water in the beaker by additions of cold water. Experiments with mixtures of air and methane have shown that when the gas has passed ten times over the hot wire and into the potassium hydroxide solution and five times over the cold wire and into the potassium hydroxide solution, all of the methane is burned and the carbon dioxide absorbed. Care must be taken to have the cooling bath at the same temperature at the end as it was at the beginning of the analysis.

### Short Method of Calibrating Burette.

A simpler method of calibrating the burette than that given earlier (Carpenter, 1923) has been devised. If the bore of each of the

TABLE I.

*Tests of Uniformity of Bore in Graduated Capillaries of Measuring Burette.*

| Position in capillary. | Upper portion of burette, readings on mercury bubble. | | | Lower portion of burette, readings on mercury bubble. | | |
|---|---|---|---|---|---|---|
| | Left end. | Right end. | Difference. | Left end. | Right end. | Difference. |
| 1 | 78.452 | 78.898 | 0.446 | 98.527 | 99.447 | 0.920 |
| 2 | 78.569 | 79.014 | 0.445 | 98.720 | 99.637 | 0.917 |
| 3 | 78.676 | 79.121 | 0.445 | 98.850 | 99.760 | 0.910 |
| 4 | 78.836 | 79.284 | 0.448 | 99.059 | 99.960 | 0.901 |
| 5 | 78.986 | 79.433 | 0.447 | 99.201 | 100.101 | 0.900 |
| 6 | 79.115 | 79.562 | 0.447 | | | |

graduated capillary sections is uniform in diameter, it is not necessary to determine the volumes for the individual graduations, but simply the volume of the entire graduated section. The uniformity of the bore is estimated by readings of the burette at the ends of a column of mercury which takes up about 0.5 per cent (*i.e.* circa 50 mm.) in length, the burette being held horizontal. The column is read at different sections of the graduated capillary and the differences between the readings at the ends of the mercury should be uniform to within 0.003 per cent. If the bore in each graduated capillary is found uniform, the weight of mercury in the burette is then obtained from the stop-cock to the beginning of the graduated capillary, then for the upper graduated section, then for the lower bulb, and lastly for the lower graduated section.

The corrections may then be computed and interpolated for the individual graduations to the nearest 0.001 per cent.
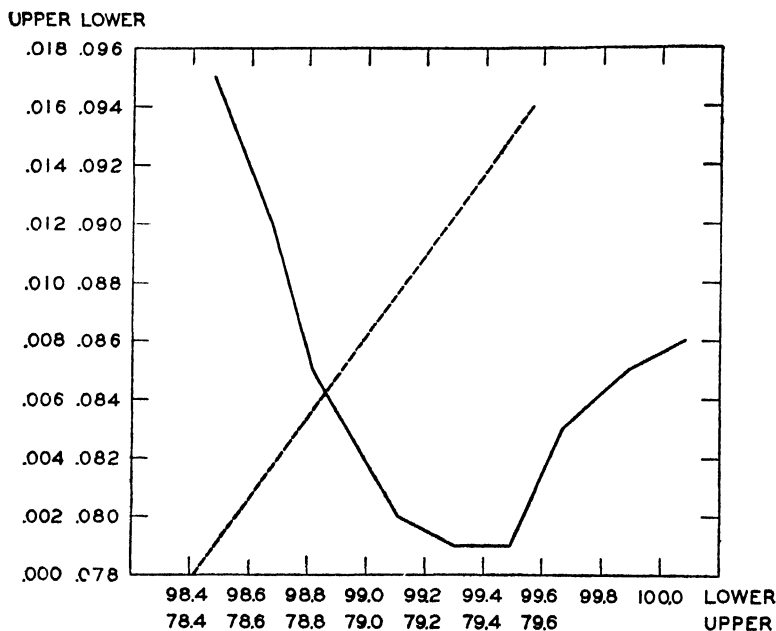


Fig. 4. Sample calibration of graduated portions of burette. The corrections for the upper portion of the burette are shown as a straight broken line from ±0.000 at the reading 78.418 to +0.016 at 79.562, because of the uniformity of the bore in this part of the burette. The scale at the extreme left of the drawing provides for interpolations for intermediate graduations of the capillary. Drawn across the broken line is the irregular full line representing corrections on the lower portion of the burette. The corrections are plotted as obtained from weighings of mercury at nine points from 98.477 to 100.083. These corrections range from +0.095 to +0.079 on the upper section and from +0.079 to +0.086 on the lower section of this portion of the burette.

A sample of the testing of uniformity of bore is shown in Table I. The upper graduated capillary showed a high degree of uniformity throughout its length. There was a range of 0.003 per cent in the measurements of a column equal to about 0.4 per cent of the total volume of the burette. On the contrary, the lower part of the bu-

rette showed an irregularity, the upper section of the capillary being of smaller bore than the lower section. A burette of this character requires, therefore, calibration for individual graduations of the lower capillary, whereas for the upper capillary one weight of mercury may be taken and the corrections interpolated for the individual graduations. Weighings have accordingly been made of mercury required to fill different parts of this burette: from the

TABLE II.

*Example of Calibration of Measuring Burette.*

| Location on graduated capillary. | Burette reading (approximate). (a) | Weight of mercury. (b) | Equivalents of mercury. ( (b) + 5.3849)* (c) | Corrected reading of burette. (78.418 + (c) ) (d) | Correction. ((d) − (a)) (e) |
|---|---|---|---|---|---|
| | *per cent* | *gm.* | | | |
| Upper portion, top. | 78.418 | 422.274 | | 78.418 | ±0.000 |
| "      "      bottom. | 79.562 | 6.2440 | 1.1595 | 79.578 | +0.016 |
| Lower portion, top and | 98.477 | 102.283 | 18.9944 | 98.572 | +0.095 |
| individual graduations. | 98.675 | 1.0375 | 0.1927 | 98.765 | +0.090 |
| | 98.817 | 0.7420 | 0.1378 | 98.902 | +0.085 |
| | 99.103 | 1.5115 | 0.2807 | 99.183 | +0.080 |
| | 99.295 | 1.0255 | 0.1904 | 99.374 | +0.079 |
| | 99.489 | 1.0450 | 0.1941 | 99.568 | +0.079 |
| | 99.672 | 1.0105 | 0.1877 | 99.755 | +0.083 |
| | 99.893 | 1.1980 | 0.2225 | 99.978 | +0.085 |
| | 100.083 | 1.0280 | 0.1909 | 100.169 | +0.086 |

* This factor (422.274 ÷ 78.418), computed from the mercury weighed in the burette between stop-cock and beginning of the graduated portion, is taken as the standard weight per unit 1.000 for the remainder of the burette; *i.e.*, for approximately 1.000 per cent of the volume of the burette. The unit equivalents of mercury in Column $c$ are volumes in the burette readings corrected according to the standard.

stop-cock to the beginning of the graduated portions (readings, 0 to 78.418; mercury, 422. 274 gm.), for the upper graduated portion, for the lower bulb, and finally for successive sections of the lower graduated portion (see Table II). The plotting of the corrections for the two graduated portions is shown in Fig. 4 and from the curve of the corrections for the lower portion Table III has been prepared.

*Arrangement for Operating by Motor.*

A motor system has been arranged for operating these gas analysis apparatus either singly or two at a time, as desired. This system consists of a ⅛ horse power, 110 volt D. C. motor, rated at 1700 R. P. M., which is geared to a rotation pulley with an extension arm. The ratio of the pulleys on the motor reduction is 4½ to 1 and on the reduction gear, 48 to 1. There is a variable resistance of 175 ohms in series with the motor to reduce the speed. Two lifting chains are fastened to the extension arm and these are carried over pulleys to points conveniently near the apparatus. The mercury reservoirs of two gas analysis apparatus may be

TABLE III.

*Corrections for Lower Portion of Measuring Burette.*

| Burette readings. | Correction | Burette readings. | Correction. | Burette readings. | Correction. |
|---|---|---|---|---|---|
| 98.477–98.496 | +0.095 | 98.746–98.774 | +0.087 | 99.200– 99.511 | +0.079 |
| 98.497–98.536 | +0.094 | 98.775–98.802 | +0.086 | 99.512– 99.557 | +0.080 |
| 98.537–98.575 | +0.093 | 98.803–98.845 | +0.085 | 99.558– 99.603 | +0.081 |
| 98.576–98.615 | +0.092 | 98.846–98.902 | +0.084 | 99.604– 99.649 | +0.082 |
| 98.616–98.655 | +0.091 | 98.903–98.960 | +0.083 | 99.650– 99.727 | +0.083 |
| 98.656–98.689 | +0.090 | 98.961–99.017 | +0.082 | 99.728– 99.837 | +0.084 |
| 98.690–98.717 | +0.089 | 99.018–99.074 | +0.081 | 99.838– 99.988 | +0.085 |
| 98.718–98.745 | +0.088 | 99.075–99.199 | +0.080 | 99.989–100.083 | +0.086 |

fastened to these lifting chains and, because of the rotation pulley, one reservoir will be lifted at the same time that the other is lowered. Thus the readings on the two apparatus may be made in succession and not simultaneously. On the other hand, if one apparatus is out of order and requires attention, it is still possible to operate the second apparatus automatically while searching for the trouble in the first. The rate of movement of the lifting chains is six times per minute, and the extent to which the mercury reservoirs are raised and lowered is 28 cm. Strieck (1928) has also developed a motor device for simultaneously lifting two mercury reservoirs of gas analysis apparatus of this type. His device differs, however, in that the reservoirs are raised and lowered not in alternate succession but both in the same direction at the same time. With his arrangement it is necessary for the operator to

watch the mercury reservoirs and to reverse by hand the direction of the motor. With our arrangement it is not necessary to control by hand and it is not necessary to watch the reservoirs. Whenever the mercury reservoir is to be raised and lowered, it is attached to the lifting chain at a fixed point so that it cannot be lifted too high and drive the mercury into the capillary connections. Theoretically, the arrangement of Strieck is better, because the mercury reservoir can be held at the limits of the raising or lowering long enough for all of the gas to pass into the pipette or into the burette. With our arrangement only a portion of the gas moves back and forth into the burette or into the pipette. But since it is not necessary to watch the arrangement or to control it by hand, one gas analysis apparatus may be in operation while readings are being made on the second. Before a reading is made after the absorption of oxygen, it is necessary to drive the gas into the pyrogallate solution five times by hand lifting, in order to insure complete removal of the last portion of oxygen.

### Notes on Use of Apparatus.

From correspondence with other workers and from visits, it was learned that there had been difficulty in obtaining satisfactory results with the apparatus, and this has been found to be due to several causes. The dead space between the gas sampler and the connection of the burette must be as short and as small as possible, because otherwise the residual nitrogen in the apparatus at the end of an analysis will not be completely swept out by the washing method, and if a portion of nitrogen is left in the connection, it reduces the oxygen content of the succeeding sample drawn into the burette.

If there is much water above the mercury in the burette at the conclusion of an analysis, a correction must be applied in the calculation of the results. Our procedure is as follows: Readings are made on the original volume, the volume after the absorption of carbon dioxide, and the volume after the absorption of oxygen. These are all readings of the *mercury* meniscus. After the last of these readings is made, a reading of the *water* meniscus is obtained since by that time the maximum amount of visible water has accumulated above the mercury meniscus. The difference between the last mercury meniscus reading and the water meniscus reading

is subtracted from the reading made on the original volume. This gives the most nearly correct initial reading for the calculations of the percentages of carbon dioxide and oxygen in the sample. The volume of water is of significance because, for every 0.05 per cent of volume due to visible water, 0.01 per cent error would result in the determination of oxygen. It is admitted that even this method does not take into account all of the water in the burette, but only the maximum amount that can be obtained by reading. We usually keep the volume of water between 0.05 and 0.10 per cent.

In Table IV is an illustration of the way air analyses are recorded and computed. Two analyses made with the Haldane-Carpenter apparatus are given, a sample of outdoor air and an aliquot sample of diluted air from a subject. Then are shown the form in which volumes are recorded after the absorption of carbon dioxide and of oxygen, the application of burette corrections to obtain corrected volumes, and the computation of percentages of carbon dioxide and oxygen in the analyses, the carbon dioxide output and oxygen deficit, and the respiratory quotient. A final reading of the burette in each analysis provides a minus correction to be applied to the initial reading to obtain the true initial volume which is the basis for the calculation of percentages of gases absorbed. The burette corrections in Table IV represent averages of several calibrations made on Apparatus 38, whereas the corrections shown in Table III and Fig. 4 are from one sample calibration of the same apparatus.

We have known of two instances in which difficulty was found in making the potassium hydroxide solution of the specific gravity of 1.55 for the preparation of potassium pyrogallate solution. It is possible to obtain the specific gravity of 1.60 with potash solution at the temperature of 15.5°, but in order to do so, the last trace of solid potassium hydroxide must be dissolved and the solution must be cooled very slowly to prevent crystallization. We have found it of advantage to pass compressed air through the solution in dissolving the last particle of potassium hydroxide and have repeatedly obtained the specific gravity of 1.55 when the solution was cooled carefully.* This specific gravity is obtained by dissolving 300 gm. of potassium hydroxide in 200 cc. of water when an 85 to 88 per cent potassium hydroxide is used.

TABLE IV.

*Sample of Form for Record and Computation of Results of Analyses of Air Samples.*

| Reading. | | Correction. | Corrected volume. | Difference | $H_2O$ | True volume. |
|---|---|---|---|---|---|---|

Outdoor air.   Apparatus 38.   Date of analysis, Dec. 4, 1928. Analyst, M. D. F.

| Reading. | | Correction. | Corrected volume. | Difference | $H_2O$ | True volume. |
|---|---|---|---|---|---|---|
| Volume. | 100.028 | | | | | |
| " | 100.029 | +0.086 | 100.115 | | 0.080 | 100.035 |
| $-CO_2$ | 99.997 | +0.086 | 100.083 | 0.032 | | |
| " | | | | | | |
| $-O_2$ | 79.132 | | | | | |
| " | 79.130 | +0.007 | 79.137 | 20 946 | | |
| " | | | | | | |
| $-H_2O$ | 79.050 | | | | | |
| | *per cent* | | | | | |
| $CO_2$ | 0.032 | | | | | |
| $O_2$ | 20.939 | | | | | |

Subject, Miss W.   Apparatus 38  Date of experiment, Dec. 4, 1928. Aliquot Sample 14.   Time 9.30 a.m.   Date of analysis, Dec. 4, 1928. Analyst, M. D. F.

| Reading. | | Correction. | Corrected volume. | Difference | $H_2O$ | True volume. |
|---|---|---|---|---|---|---|
| Volume. | 99.977 | | | | | |
| " | 99 975 | +0.086 | 100.061 | | 0.069 | 99.992 |
| $-CO_2$ | 99.061 | | | | | |
| " | 99.063 | +0.081 | 99.144 | 0.917 | | |
| $-O_2$ | 79.228 | | | | | |
| " | 79.227 | +0.008 | 79.235 | 19.909 | | |
| " | | | | | | |
| $-H_2O$ | 79 158 | | | | | |

| Outgoing air | | CO₂ output. | | O₂ deficit. | |
|---|---|---|---|---|---|
| | *per cent* | | *per cent* | | *per cent* |
| $CO_2$ | 0.917 | Out. | 0.917 | | 20.978* |
| $O_2$ | 19.911 | In. | 0.030 | Out. | 19.911 |
| | 20.828 | | 0.887 | Deficit. | 1.067 |
| | 100.000 | | | | |
| $N_2$ | 79.172 | | | | |

$$\frac{CO_2}{O_2} = \frac{0.887}{1.067} = 0.831 \text{ R.Q.}$$

* Computed by formula; 79.030: per cent $N_2$ :: 20.940 : x.

It is our practice to prepare potassium pyrogallate[2] in such quantities that one or more containers will be required to fill the potassium pyrogallate pipette.   The solution is stored in bottles with narrow mouths, which are closed with cork stoppers and then sealed with hot paraffin.   We never prepare potassium pyrogallate in a large quantity, stored in one container, and we never use wide-mouthed bottles, as is frequently the practice.   Storage in small containers is more convenient and, in case the solution spoils, it also prevents the loss of a large quantity at once.   The sealing with paraffin insures against leakage, which would occur if the opening of the bottle were not absolutely tight because of the diminished pressure due to absorption of oxygen.

The water in the jacket surrounding the burette and compensator tube is usually stirred by means of compressed air which is admitted through a glass tube extending to the lower part of the jacket.   If this tube is of small diameter or drawn out at the end, a fine stream of bubbles results, which is cooled by the evaporation of water.   As these bubbles leave the top of the water jacket, they cool the capillary at the upper part of the burette and cause condensation of moisture in the capillary, and bring about moisture and temperature conditions inside the capillary which are variable, and these prevent accurate analysis.   It is best to have the compressed air pass through a moistener outside of the water jacket, and then to admit it through a tube large enough ($\frac{1}{4}$ inch diameter) to prevent the bubbles of air from forming a fine stream.

When the atmospheric pressure is rapidly changing, as on a day with variable winds, it is difficult to obtain settings of the levels of the potassium hydroxide solution because they shift with every change of external pressure.   This difficulty may be avoided in the following way: A one-hole rubber stopper is inserted into the mouth of the levelling bulb of the potassium hydroxide pipette and in this stopper is placed a one-way glass stop-cock.   When the adjustment of the solution is to be made, it is first made roughly, and then the stop-cock is closed.   Final adjustment of the levels is then made independent of external pressure.   *The stop-cock in the rubber stopper in the mouth of the potassium hydroxide levelling bulb must always be open when the potassium hydroxide solution is raised*

---

[2] We use No. 215 pyrogallol, Eastman Kodak Company, Rochester, N. Y.

*and lowered* during the absorption of carbon dioxide, for the reason that if the stop-cock is closed during the movement of the potassium hydroxide solution, the solution will be driven into the compensator tube.

The perfect functioning of the gas analysis apparatus depends upon the maintenance of extreme sensitivity. The readings of the burette are made to approximately 0.001 per cent which is equivalent to a change in pressure 0.008 mm. of mercury or about 0.1 mm. of water. If it happens that the ports of the stop-cocks, . $O$ and $D$ (these and all further references are to Fig. 1), are partly closed with grease, it is easy to move the gas back and forth when the mercury bulb, $N$, is raised and lowered, but the final adjustment cannot be made accurately because of the formation of a slight film of grease over the openings of the ports. A globule of mercury or a drop of liquid in any part of the capillaries will prevent accurate final adjustment. Bubbles of air in a rubber connection or in the rubber tubing of the potassium hydroxide pipette, $E$, will prevent accurate adjustment for reading. None of these conditions may be apparent until the analyses of outdoor air are made and then poor results will be secured. Occasionally, it is found that constant readings cannot be obtained when apparently all of the oxygen is absorbed. This may be due to a leak or to a lack of compensation. The presence of a leak in one or both of the stop-cocks, $O$ and $D$, of the burette, $K$, and the potassium hydroxide pipette, $E$, is most easily determined by closing stop-cock $D$ of the potassium hydroxide pipette, $E$, and raising the mercury levelling bulb, $N$, until the gas in the burette, $K$, and adjoining capillaries is under several cm. pressure, allowing it to remain so for several minutes. Readings made of the burette at the beginning and end of the test should be the same to within 0.002. A test of stop-cock $O$ alone can be made in the same manner by closing the stop-cock and putting the gas in the burette, $K$, alone under pressure. The same agreement in results should be obtained as above.

A diminished volume in a test of the apparatus indicates a leak or a lack of compensation. If during these tests, the levels of the potassium hydroxide or of the potassium pyrogallate go down, then stop-cock $D$ is leaking, and it should be greased[3] until a test of this

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[3] We have found "Lubriseal," distributed by the Arthur H. Thomas Company, Philadelphia, satisfactory for the lubrication of stop-cocks in this apparatus.

character shows no change in the levels of the liquids. A decrease of volume in the burette, $K$, after these tests may not always indicate a leak, but may arise from a lack of compensation. To prove this, a test should be made in the same manner, but by lowering the mercury bulb, $N$, so that the gas is under diminished pressure. If there is a leak between the level of mercury in the burette and that of the potassium hydroxide in the capillary of the pipette, the volume will increase, but if there is inadequate compensation the volume will decrease as it did in the test with increased pressure. If the trouble, then, is not leaks, but lack of compensation, all parts of the apparatus must be examined to see whether there are bubbles of air in the potassium hydroxide, or globules of mercury or water in the capillaries, or too much grease in the ports of the stop-cocks.

Another cause of lack of compensation or sensitivity of adjustment is sediment in the potassium hydroxide which may collect in the capillaries of the potassium hydroxide pipette, $E$, and the compensator capillary, $J$. The use of 5 per cent solution of potassium hydroxide is recommended and it should be renewed as soon as any sediment forms. The solution should always be water-clear.

Inability to obtain constant readings may also be caused by a leak in the compensator stop-cock, $H$. This does not occur often, but still so frequently that possibility of a leak at that point should always be eliminated when the apparatus is not functioning properly. A test for this leak can be made in the following manner: The burette is read and the stop-cock, $D$, of the potassium hydroxide pipette, $E$, is closed. The levelling bulb, $P$, of pipette $E$ is lowered and suspended by wire or cord on the bracket, $Q$, which supports the levelling bulb, $P$, the lowering being done so far as possible without letting air bubbles into the rubber junction of the capillary tee, $G$, and the Y-shaped tube, $S$, of the pipette, $E$. Bulb $P$ is left suspended for several minutes, and then is raised to its former position, the stop-cock, $D$, of the potassium hydroxide pipette, $E$, is opened and a reading of the burette is again made. The volume should be unaltered if the compensator stop-cock, $H$, is tight. If it has leaked during the test, the final volume will be smaller. The reason for this is, that when there is an increase in volume

in the compensator, *B*, more pressure is required to bring the potassium hydroxide levels to the marks and increased pressure on the gas in the burette, *K*, results in diminished volume.

A good test of sensitivity or compensation is to set the potassium hydroxide levels in *J* and *C* with the stop-cock, *H*, closed. The stop-cocks, *O* and *D*, are left open. After the levels have been exactly set, the apparatus is not used, but left for 5 to 10 minutes. Usually there is a change in the potassium hydroxide levels in *J* and *C* due to a change in external temperature, which is either falling or rising. If the external temperature is falling, the levels will rise in both *J* and *C* and, if the apparatus is sensitive and the capillarity is the same for *J* and *C*, the potassium hydroxide levels will rise equal distances. On the contrary, if there is cause for sluggishness, as, for example, sediment in the potassium hydroxide in the capillary, *J*, then there will be an unequal rise in the two capillaries, which will indicate inadequate compensation. This test is to be strongly recommended, and if it is found that there is not enough change in temperature to produce changes in levels, these changes may be brought about artificially by the addition of cold or warm water to the water jacket, *A*.

### SUMMARY.

A description is given of changes in the compensating system of a gas analysis apparatus for outdoor and respiratory chamber air, together with the method of adjustment for adequate compensation, of a modified pipette for potassium pyrogallate, of an alteration in the combustion unit for methane, of a short method of calibrating the measuring burette, and of an arrangement for operating by motor. Remarks are included on the use of the apparatus with special reference to dead space in sampling, water in burette, specific gravity of potassium hydroxide solution, and preparation and storage of potassium pyrogallate, suitable size of tube for compressed air used for stirring in water jacket, variable atmospheric pressure, and tests for tightness and adequate compensation.

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# SOME CHEMICAL INVESTIGATIONS OF EMBRYONIC METABOLISM.

## III. A STUDY OF THE NITROGEN DISTRIBUTION IN THE DEVELOPING HEN'S EGG BY THE MODIFIED VAN SLYKE PROCEDURE.

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The excellent review by Needham in 1925 (1), entitled "The Metabolism of the Developing Egg," was a stimulus for a large number of investigations of embryonic metabolism. Needham indicated two serious defects in the work of the early investigators. In the first place, the results may not be statistically sound. In many cases an insufficient number of eggs was used for each period and conclusions cannot be drawn from the investigation. A second problem, which is even more important than the first, is presented by the indefiniteness of morphological boundaries. When the egg has been separated into various fractions the investigator has seldom been explicit in his statements concerning what membranes and what fluids were included in each fraction. As a result correlations are not uniform and confusion enters. The same criticisms apply to some of the work done since Needham so clearly pointed out the defects mentioned above.

The present investigation was suggested by earlier work on embryonic metabolism. It seems possible that a correlation can be made between the earlier findings of nucleic acid synthesis and changes in other nitrogenous constituents. The investigation, which is reported in this paper, consists of a complete study of the changes in the distribution of nitrogen which occur from day to day as indicated by the method of Van Slyke as modified by Plimmer and Rosedale (2). There has been no complete investigation reported in the literature and it seemed advisable that such a study should be made. The Van Slyke method enables one to

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study various types of nitrogen that cannot be studied by any other method.   Further investigations and a general discussion are included in the succeeding paper of this series.

The nitrogen metabolism of the developing hen's egg had been investigated by only a very few workers before Needham (1) published his review.   A brief summary of the investigations of the amino acids and other nitrogenous constituents may not be out of place here.

Abderhalden and Kempe (3) in an attempt to throw light on the protein metabolism of the developing egg, estimated the tyrosine, glutamic acid, and glycine at three different stages.   They succeeded only in showing that the total amount of these substances remained unchanged.   Levene (4) showed that free monoamino acids were present during development    The tryptophane content of the egg at the beginning of incubation was estimated with consistent results (5, 6).   Cho (7) found quantitative changes in tryptophane, cystine, and tyrosine.   Sendju (8) found a gradual decrease in tyrosine during development and a great reduction in the tryptophane at the time of appearance of blood pigments on the 3rd day of incubation    Later, when the bile pigment appeared, there was a further reduction of tryptophane, indicating its use in the formation of these pigments.   Free and combined purine nitrogen increased according to Sendju, while the hexone bases and the nitrogen not precipitated by phosphotungstic acid remained practically constant.   In a later investigation, Sendju (9) found that the cystine content of the whole egg, as well as in each of the three fractions, yolk, white, and embryo, gradually decreased.   Cho (7) found a slight decrease in cystine, while Plimmer and Lowndes (10) found practically no change.

Sendju (8) reported that the arginine, histidine, and lysine remained practically constant.   Russo (11) estimated the hexone bases on the fresh egg, at the 10th day and at the 18th day, and found an 18 per cent decrease during that period, most of which was accounted for by the loss in arginine, while the lysine remained constant.   He found an increase in purine nitrogen, from which he concluded that there was a genetic relationship between the hexone bases, arginine and histidine, and the purine base nitrogen.   Plimmer and Lowndes (10), using the Van Slyke distribution method, have shown that there was an increase of 2 per cent in the diamino nitrogen, 50 per cent of which could be accounted for by the increase in the arginine.   The monoamino acid nitrogen in the filtrate from the bases decreased approximately 4 per cent during development, while the amino nitrogen of that fraction decreased correspondingly.   Their figures indicated a very slight decrease in lysine and they stated that the histidine values were not dependable by this method.   Their series was not complete since examinations were made only of the fresh egg, at the 15th day, and of the hatched chick so that definite conclusions cannot be drawn.   However, theirs is the only

investigation of nitrogen distribution during the development of the hen's egg.

Tomita (12), Aggazzotti (13), and Hepburn (14) have all three concerned themselves with the non-protein nitrogen. Tomita and Aggazzotti found an increase in the non-protein nitrogen of the whole egg during development. Hepburn studied only fresh eggs and storage eggs. Wladimirov and Schmidt (15) found that the non-protein nitrogen of the egg white increased in proportion to the rise in total nitrogen during development. Nakamura (16) found an increase in the total "rest nitrogen," both that which was precipitated by phosphotungstic acid and that not precipitated. Needham (17) estimated the non-protein nitrogen quantitatively throughout the period of development. Its rise followed a regular curve and exhibited two peaks, one on the 5th day and the other on the 13th day. The peaks came immediately after the peaks of protein absorption.

Fiske and Boyden (18), in a very excellent investigation, estimated the uric acid, total nitrogen, residual nitrogen, creatine, and amino acid content of the allantoic fluid of chick embryos during the first 2 weeks of development. Creatine was present in the embryonic tissues as early as the 8th day. This is contrary to the results of Mellanby (19) who was unable to find creatine before the 12th day and then in traces only. After the 12th day he found a gradual increase up to the time of hatching and a tremendous increase in the 1 day chick as compared with the new born. Sendju (8) demonstrated the presence of both creatine and creatinine in the fresh egg and found a marked increase, especially of creatine, during the developmental period. His results are not in accord with the results either of Mellanby or Fiske and Boyden.

Needham (20) who, in his very thorough investigations of energy sources in ontogenesis, studied the urea, uric acid, and ammonia of the egg during the whole period of development, demonstrated that there was an intensive production of urea from the 5th to the 9th day. After the 9th day the formations of urea failed to keep pace with the growth and differentiation of the embryo. There was a period of intensive uric acid production from the 7th to the 11th day. This period is 2 days later than the period of intensive production of urea and after reaching this peak the uric acid did not keep pace with the growth of the embryo. By the 10th day 95 per cent of the total nitrogen excreted was uric acid nitrogen, while most of the nitrogen was excreted in the form of urea up to the 7th day. Ammonia, in absolute amounts, steadily increased during incubation and the intensity of production reached a peak on the 4th day.

Idzumi (21) in a chemical and serological examination of the hen's egg during development found an increase in the "residual" and amino nitrogen, while the total nitrogen remained constant.

Sagara (22) studied the changes in the free hexone bases and purines in the embryo. He found that the purines, arginine and lysine, reached a maximum on the 14th day, while the histidine remained constant.

Among the other nitrogenous substances which have been the subject of special investigations are the purines which have been consistently shown

to increase (23)   Sharpe (24) estimated choline and found a steady decrease from the beginning.  Nakamura (16) found that both free and combined choline increased gradually in the embryo, while the bound choline reached a peak on the 9th day in the yolk and decreased thereafter   Burns (25) found a continued increase in the guanidine content until the 12th day of incubation   Thereafter there was a marked decrease followed by a period of oscillation and finally a slight but steady rise.

### EXPERIMENTAL.

The eggs of White Leghorn hens were placed in the incubator on the 2nd day after they were laid.   The hens were kept on a constant diet and the eggs were incubated as nearly as possible under the standard conditions suggested by Murray (26); that is, the humidity was constant and there was continuous ventilation. The eggs were aired 15 to 20 minutes each day and turned once a day.   The range in weight was 50 to 67 gm. (average 57 gm.). The loss in weight of those which were incubated for the entire period was 3.5 to 5 gm. (average 4 gm.).

Five eggs were selected each day for investigation.   They were examined under an electric light for fertility.   Each egg was then broken into an evaporating dish and the embryo examined to determine its age.   If it was not at the proper stage of development it was discarded.   By this means synchronously developed eggs were obtained.   The five eggs were then put into 1500 cc. of absolute alcohol, stirred thoroughly, and allowed to stand 24 hours.   After the 6th day it was necessary to cut the embryos into small pieces with shears and after the 14th day it was found best to put the whole egg through a food chopper.   The shell was always included unless otherwise designated.

After standing in alcohol for 24 hours the insoluble material was filtered off, washed with alcohol and ether, quantitatively transferred to a Soxhlet apparatus, and extracted for 8 hours with absolute alcohol.   It was then further extracted with ether and finally dried in a vacuum desiccator over sulfuric acid for 24 hours. This dried material was easily reduced to a fine powder in a mill and preserved in this condition in bottles sealed with paraffin.

The nitrogen distribution in the material obtained by the above technique was determined by the Van Slyke (27) method as modified by Plimmer and Rosedale (2) with the further changes indicated below.

1. 15 gm. of the material were hydrolyzed for 36 hours with 20 per cent hydrochloric acid. The *acid-insoluble melanin*, which is usually not removed at this point, was filtered off after the solution was cold, washed until the washings were clear, and analyzed for total nitrogen. No tests were made from time to time to follow the progress of the hydrolysis. After the hydrochloric acid was removed the residue was made up to 500 cc., the total nitrogen determined, and added to the acid melanin nitrogen. The Kjeldahl-Gunning method was used for all total nitrogen determinations.

2. The Van Slyke procedure for amide nitrogen was modified according to the technique of Plimmer and Rosedale (2) with the following changes. The Claisen flasks used were 2 liter Claisen flasks and the tube attached to the end of the condenser was a Folin aeration tube. The fine holes in the bulb, when covered well with the water and standard acid, prevented any loss due to splashing.

The volume in the distillation flasks was reduced to 50 cc. This part of the Plimmer and Rosedale procedure is distinctly different from that recommended by Van Slyke who specifically states that the temperature for the distillation should be 60° and the time of distillation 30 minutes. In this investigation the temperature ranged from 40 to 60° and the time of distillation from 30 to 45 minutes.

3. The *alkali-insoluble melanin or humin* was removed by filtration and the total nitrogen determined according to the technique followed by Plimmer and Rosedale (2). By this procedure the concentration of the filtrate and washings was avoided and the bases can be precipitated at once. This is in marked contrast with the original Van Slyke procedure in which the volume of the combined filtrate and washings was always large and considerable time was necessary for the concentration.

4. The Van Slyke procedure was followed for precipitation of the bases. The method of removal of the bases was changed and the filtration carried out on Jena glass funnels with ground glass plates. If funnels with fine pores were used no trace of the precipitate passed through and a second filtration through paper was not necessary as in the regular Van Slyke procedure. The washing

was carried out according to the Plimmer and Rosedale (2) technique except that the wash solution was cooled to zero degrees.

5. The precipitate was dissolved in normal sodium hydroxide. Usually three portions of 10 cc. each were sufficient to dissolve the precipitate; however, five portions were always used to avoid any possible loss in the pores of the filter.   The solution and washings were made up to 100 cc.   The several estimations which follow were equally well made in the presence of phosphotungstic acid and bumping did not occur in the Kjeldahl operation, if the oxidation was carried out slowly according to the directions of Plimmer and Rosedale.   The arginine was determined by the Plimmer (28) and Koehler (29) modifications of the Van Slyke (27) procedure.

6. The amino nitrogen was determined in the solution of the bases in the micro apparatus of Van Slyke (30).   The determination was continued for 30 minutes, since Van Slyke has shown that a longer time was required for the total amino nitrogen of the lysine.

7. The cystine content of the solution of the bases was estimated by the procedure of Plimmer and Lowndes (31) without removal of the phosphotungstic acid.   The following changes were found essential.   50 cc. of a 20 per cent barium chloride were required for complete precipitation instead of the usual 10 cc. and complete precipitation did not occur in 24 hours, but 4 or 5 days were required.   The solutions were kept in an ice box for this length of time.   The precipitate was filtered on Jena glass crucibles with ground glass plates and the tedious procedure of ignition of the filter paper was avoided.   The cystine content was calculated from the barium sulfate obtained.

8. The total nitrogen and amino nitrogen of the filtrate were determined according to Van Slyke.   The calculations in this investigation were made in the usual manner for the following forms of nitrogen: acid-insoluble melanin, amide, humin, amino nitrogen of the filtrate, non-amino nitrogen of the filtrate, amino nitrogen of the bases, non-amino nitrogen of the bases, arginine, histidine, lysine, and cystine.   The results are given in Table I in percentage of the total nitrogen of the sample, which is the usual manner of recording the results obtained by the Van Slyke procedure.   The corrections for the calculations of the amino acids recommended by Van Slyke have not been applied.

These corrections have been omitted because of the fact that they are small and also due to the fact that several investigators have found them inaccurate (32). Their inclusion would not alter the conclusions which may be drawn from this investigation.

<p align="center">DISCUSSION.</p>

A careful study of the brief historical review of the nitrogen metabolism of the developing egg, presented in the early part of this paper, is not necessary to convince the observer that a great deal of further study is required before definite correlations can be made. Although many of the differences in the results obtained by the various investigators may be explained by differences in the methods used, some may be explained by the fact that too few observations have been made, or that too few eggs have been used in making these observations; others may be explained by the fact that in some cases the shell and its membranes were not included; still others may be explained by the fact that the eggs of different breeds of chickens may differ in chemical composition and in the chemical changes occurring during development.

The only possible means of overcoming these difficulties is for the observer to state definitely and clearly the exact conditions under which the observations have been made. The conditions of incubation, morphological boundaries, methods of estimation, kind of eggs, number of eggs, etc., are some of the considerations which should be carefully mentioned.

As previously stated, the interesting information obtained in the incomplete investigation of Plimmer and Lowndes (10), of the nitrogen distribution in the hen's egg during development, necessitated a completion of their investigation over the entire period of 21 days. A study of the table will show that the results of these investigators have, in general, been confirmed. But it will also show that a larger number of eggs have been used for each observation. Furthermore, the series should be more accurate since it is complete from the fresh egg through the entire period of development of 21 days. Plimmer and Lowndes only made observations on the fresh egg, at the 15th day, and on the hatched chick.

There have been many criticisms of the Van Slyke method for the determination of the nitrogen distribution and although these

may be justified there has been no other method offered to replace it. The values obtained may not be exact but they are uniform and in quite close agreement with those obtained by other methods, at least, for some of the amino acids. It is also the only method

TABLE I.

*Values Are Expressed in Per Cent of Total Nitrogen.*

| Day of test | Acid melanin | Amide | Humin | Total N of filtrate | Amino N of filtrate | Total N of bases | Amino N of bases | Non-amino N of filtrate | Non-amino N of bases | Arginine N | Cystine N | Histidine N | Lysine N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0* | 0 88 | 8 69 | 0 90 | 64 05 | 61 40 | 26 30 | 13 96 | 2 65 | 12 34 | 14 53 | 0 80 | 1 82 | 8 93 |
| 1* | 0 82 | 8 34 | 1 14 | 63 83 | 61 22 | 26 12 | 13 40 | 2 61 | 12 72 | 14 45 | 0 93 | 2 53 | 8 60 |
| 2 | 0 97 | 9 12 | 0 96 | 63 38 | 61 01 | 26 58 | 13 73 | 2 37 | 12 85 | 14 18 | 1 10 | 3 36 | 8 05 |
| 3 | 1 00 | 9 55 | 1 18 | 63 05 | 60 35 | 26 20 | 14 40 | 2 70 | 11 80 | 13 55 | 0 89 | 2 40 | 9 35 |
| 4 | 0 98 | 7 58 | 1 61 | 61 00 | 57 61 | 26 75 | 13 72 | 3 39 | 13 03 | 14 27 | 0 75 | 3 46 | 8 26 |
| 5 | 0 81 | 6 84 | 0 89 | 62 54 | 60 35 | 27 75 | 14 24 | 2 19 | 13 51 | 14 72 | 0 91 | 3 31 | 8 65 |
| 6 | 0 95 | 8 46 | 1 01 | 64 30 | 62 50 | 26 38 | 13 62 | 1 80 | 12 76 | 14 35 | 0.74 | 3 06 | 8 91 |
| 7 | 0 90 | 9 00 | 1 20 | 63 81 | 61 81 | 26 05 | 13 95 | 2 00 | 12 10 | 14 10 | 0 82 | 1 98 | 8 95 |
| 8 | 1 01 | 9 01 | 1 06 | 64 05 | 60 10 | 26 40 | 14 72 | 3 95 | 11 68 | 14 12 | 0 91 | 1 98 | 9 20 |
| 9* | 1 01 | 9 08 | 1 22 | 63 57 | 61 70 | 25 73 | 14 30 | 1 87 | 11 43 | 13 65 | 0 85 | 1 60 | 9 46 |
| 10 | 1 04 | 8 44 | 1 15 | 62 25 | 58 72 | 27 05 | 14 08 | 3 53 | 12 97 | 13 25 | 0 72 | 4 74 | 8 53 |
| 11 | 0 92 | 8 97 | 1 18 | 62 50 | 58 00 | 25 96 | 13 67 | 4 50 | 12 29 | 13 40 | 0 85 | 3 23 | 8 40 |
| 12 | 0 95 | 8 69 | 1 12 | 63 25 | 58 66 | 25 92 | 14 63 | 4 59 | 11 29 | 13 87 | 0 81 | 1 12 | 9 10 |
| 13 | 1 03 | 7 58 | 1 18 | 61 50 | 55 95 | 27 84 | 15 11 | 5 55 | 12 73 | 13 88 | 0 69 | 3 64 | 9 63 |
| 14 | 1 03 | 9 14 | 1 69 | 61 85 | 56 65 | 26 22 | 14 83 | 5 20 | 11 39 | 13 78 | 0 70 | 1 35 | 10 48 |
| 15 | 0 94 | 8 88 | 1 49 | 63 95 | 59 40 | 25 93 | 14 00 | 4 55 | 11 93 | 13 32 | 0 93 | 3 01 | 9 60 |
| 16* | 1 08 | 9 21 | 1 42 | 62 01 | 55 11 | 26 47 | 16 67 | 6 90 | 9 80 | 13 70 | 0 74 | 2 51 | 9 61 |
| 17 | 0 94 | 8 93 | 1 78 | 61 75 | 55 49 | 26 15 | 14 50 | 6 26 | 11 65 | 14 42 | 0 78 | 2.19 | 9 76 |
| 18 | 1 05 | 9 36 | 1 81 | 61 52 | 55 36 | 27 79 | 14 35 | 6 17 | 13 44 | 15 28 | 0 72 | 2 76 | 8 92 |
| 19 | 0 93 | 7 98 | 1 88 | 61 60 | 55 59 | 27 92 | 14 40 | 6 01 | 13 52 | 15 70 | 1 01 | 1 08 | 10 12 |
| 20 | 0 99 | 8 22 | 1 65 | 62 00 | 55 82 | 28 25 | 15 07 | 6 18 | 13 18 | 15 82 | 0 65 | 1 69 | 10 12 |
| 21* | 0 80 | 8 50 | 2 32 | 61 00 | 54 74 | 27 25 | 13 78 | 6 26 | 13 47 | 16 43 | 0 90 | 1 81 | 8 72 |

* The values on these days are averages of four or more analyses

available for the determination of the general distribution of nitrogen in a protein hydrolysate.

However, in order to have more accurate data concerning the changes which occur in some of the amino acids during the development of the hen's egg, other methods have been used and the results will be reported in the following paper of this series. A

more detailed discussion of the results obtained by the different methods is also given in that paper.

The total nitrogen in 15 gm. of the dried material was not constant from day to day. Attempts were made to keep the conditions uniform, but these differences may be attributed to several causes. Although defatted and dried under the same conditions all the fat may not have been removed in some cases, the material may have varied in moisture content or finally, the shells may have been much heavier in some eggs. The last is the most probable reason for it is well known that even when hens are kept on uniform diets, there is a very considerable variation in the shell structure.

The modification of the Van Slyke method described here is much simpler than the original method and gives equally good results. It will be noted that the technique of Plimmer and Rosedale (2) has not been followed in detail. The procedure used for the estimation of the amide nitrogen and the precipitation of the humin, or alkali melanin, is a distinct improvement, while the modification of the apparatus by Koehler (29) for the determination of arginine is a very great improvement.

Plimmer and Rosedale (2) have discussed the advantages of making the individual determinations in the presence of phosphotungstic acid. However, in the estimation of cystine, it was impossible to get the barium sulfate to precipitate by use of the usual amount of barium chloride. If a large excess of barium chloride was used the method gave consistent results, although it was not nearly so satisfactory as could be desired. Cystine estimated by this method remained constant throughout the entire period which was quite significant in view of the fact that the sulfates of the allantoic fluid increase markedly (33).

In some of the forms of nitrogen the changes are quite striking while in other cases there is remarkable constancy throughout the entire period of incubation. The acid melanin nitrogen, the amide nitrogen, and the cystine nitrogen remain practically constant. Although the tabulated data show a tendency for the histidine nitrogen to decrease, the daily fluctuations are such that no definite conclusion is justified from these results. The humin nitrogen gradually increases while the total nitrogen of the filtrate shows a distinct decrease. One of the most outstanding changes

is the large increase in the non-amino nitrogen of the filtrate with the corresponding decrease in amino nitrogen.   It is possible that this increase is due to the fact that the chick embryo burns protein during the period of development and uses the amino nitrogen with the formation of other forms such as ammonia, urea, and uric acid (34).   The total amino and non-amino nitrogen of the bases increase slightly in each case.   The arginine presents a very interesting curve.   There is a slight but distinct decrease in the middle of the period and at the end of the incubation period there is a rapid rise even above the amount present at the beginning.   This is contrary to the results of Plimmer and Lowndes (10) who found a consistent increase.   However, their observations were too few to complete the curve.   The lysine increased slightly at the end of the period

## SUMMARY.

1. A complete study has been made of the nitrogen distribution in the developing hen's egg throughout the entire incubation period of 21 days.   The large numbers of eggs used and the study of the daily changes that occur throughout the whole period make this a much more extensive series of data than any previously reported.

2. There are no distinct changes in the acid melanin, amide, or cystine nitrogen.

3. In the fraction precipitated by phosphotungstic acid there is a small increase in the total, amino, and non-amino nitrogen.

4. There is a decrease of 3 per cent in the total nitrogen of the filtrate, but the most decided change that occurs is the increase in the non-amino nitrogen of the filtrate from about 2 per cent to a value above 6 per cent, with a corresponding decrease in the amino nitrogen.

5. The arginine nitrogen presents an interesting curve with a fall in the middle and a much more evident rise at the end of the period of incubation.   The data show a tendency for the histidine nitrogen to decrease, but the daily fluctuations are such that no definite conclusion is justified.   There is a slight increase indicated for the lysine nitrogen.

6. It should be emphasized that the changes in arginine, histidine, and lysine represented by the data obtained by this

method may not be an indication of the actual changes which occur. It should be kept in mind that the material from the whole egg obtained by the procedure described is not pure protein and may contain many interfering substances which would be precipitated by phosphotungstic acid. A further investigation of this fraction will be reported in the next paper of this series.

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# IRON IN NUTRITION.

## VIII. THE INEFFECTIVENESS OF HIGH DOSES OF IRON IN CURING ANEMIA IN THE RAT.*

BY J. WADDELL, H. STEENBOCK, AND E. B. HART.

WITH THE COOPERATION OF EVELYN VAN DONK.†

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison.*)

Clinically, it has been the practice for many years to treat certain anemias, particularly those of children, with large doses of iron salts. Very large amounts have often given beneficial results where small doses were ineffective. These results might be interpreted as a stimulating effect on hemoglobin production by the high iron intake, particularly since the iron was fed in amounts greatly in excess of the body's need for this element.

Last year it was reported (1) from this laboratory that inorganic iron salts when added to a basal diet of whole milk at a level of 0.5 mg. of Fe daily (6 days per week) were ineffective in curing a nutritional anemia in rats. Although it was not reported at that time, it was, however, observed that the same iron salts fed at a level of 2 mg. of Fe daily, stimulated hemoglobin production. These iron salts, it is to be noted, had all been prepared from the same standard iron wire with pure reagents.

In Chart I we present the results obtained with one of several litters of animals that received this supplement of iron in these early experiments. These experiments showed that the animals were able to maintain a fairly high hemoglobin level for a comparatively long time. Although many of the animals eventually showed some falling off in the hemoglobin and some died, the results were much better than had been obtained when only 0.5 mg.

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of Fe was fed. It was apparent that either the increased intake of iron was causing this increased hemoglobin production or that there was present in our iron solutions some contaminant which brought about a greater response. The demonstration (2) that
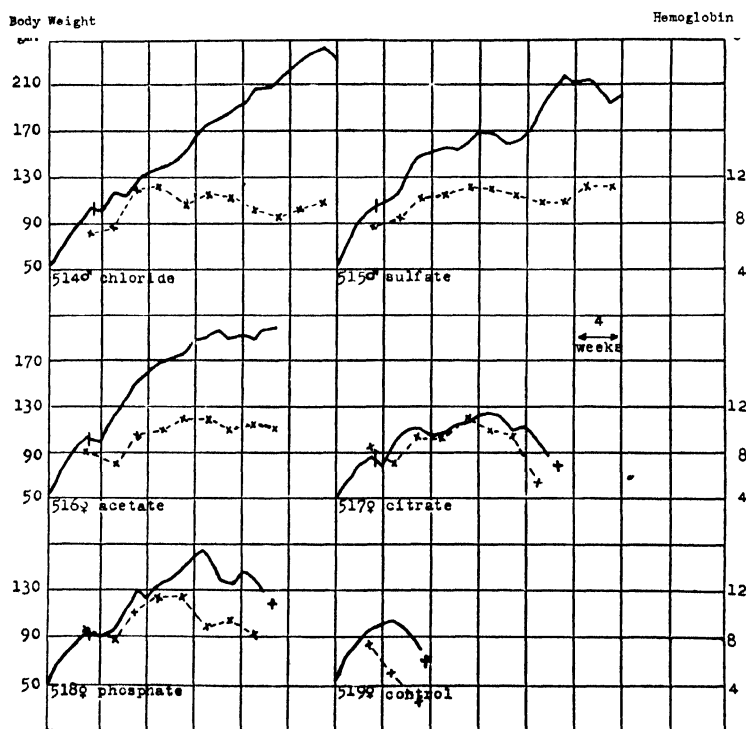


Chart I. In this chart are shown the results secured when there were added to the basal diet of cow's whole milk 2 mg. of Fe 6 days per week from the five ferric salts. These iron salts had previously been shown to be ineffective at the level of 0.5 mg. of Fe six times per week. In this and the following charts the solid line represents body weight, the broken line hemoglobin values in gm. per 100 cc. of blood. The line across the curve of body weight indicates the point at which the addition was made to the basal diet; the dagger at the end of the curve denotes death.

copper functions in the formation of hemoglobin in the body of the rat suggested that the beneficial effects of the higher dosage might have been due to the presence of small amounts of this element in our iron wire.

In view of these findings we decided to investigate the hematopoietic action of even higher doses of iron, especially purified with regard to copper.

### EXPERIMENTAL.

Since we had already shown that there was no material difference in the ability of any one of five salts of iron to supplement a basal diet of whole milk, we made use of a specially purified solution of only one; viz., ferric chloride.[1]  This solution was prepared
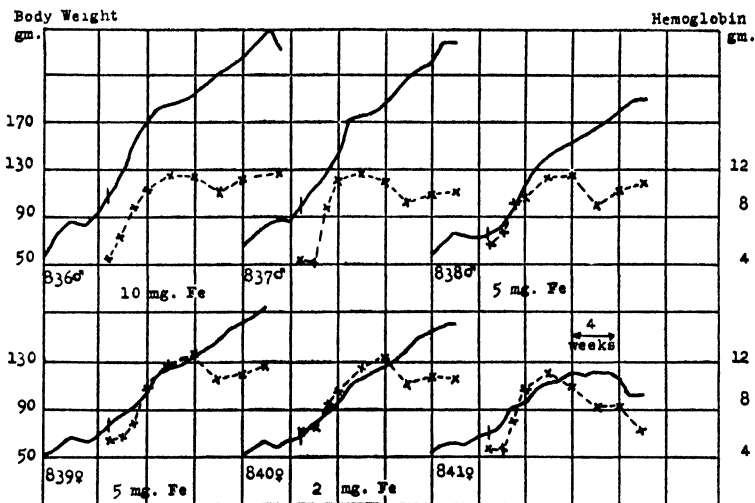


CHART II. Here are presented the results secured when there were added to the basal diet of cow's whole milk three levels of iron from the *unpurified* ferric chloride.

as we have already described ((1) p. 784) except that the iron wire was first dissolved in dilute (1:1) HCl and after dilution and heating was treated with hydrogen sulfide to remove any copper present.   After the CuS had settled out, the solution was filtered and the filtrate boiled to expel excess of the hydrogen sulfide and then concentrated on a sand bath.   After this the treatment with nitric acid and subsequent procedure was as we have described.

[1] We received cooperation in the preparation of this solution from Dr. C. A. Elvehjem of this laboratory.

TABLE I.

*Average Values for Hemoglobin and Body Weight of Animals Receiving Various Levels of Iron from Two Solutions of Ferric Chloride.*

| | 2 mg. Fe. | | 5 mg. Fe. | | 10 mg. Fe. | |
|---|---|---|---|---|---|---|
| | Body weight. | Hb | Body weight. | Hb | Body weight. | Hb |

Unpurified $FeCl_3$.

| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
|---|---|---|---|---|---|---|
| At time of addition. | 92 (7) | 6.60 (7) | 86 (4) | 6.13 (4) | 91 (4) | 5.02 (4) |
| After addition. | | | | | | |
| 2 wks. | 109 (7) | 7.33 (7) | 109 (4) | 8.65 (4) | 131 (4) | 10.02 (4) |
| 4 " | 126 (7) | 8.82*(7) | 136 (4) | 11.43*(4) | 170 (4) | 11.63*(4) |
| 6 " | 129 (7) | 10.39*(7) | 143 (4) | 11.86*(4) | 169 (4) | 11.27*(4) |
| 8 " | 138 (7) | 9.50*(7) | 154 (4) | 11.54*(4) | 186 (4) | 9.80*(4) |
| 10 " | 152 (7) | 8.96*(7) | 168 (4) | 10.03*(4) | 203 (4) | 8.55*(4) |
| 12 " | 156 (7) | 9.16*(7) | 174 (4) | 10.39*(4) | 211 (4) | 8.22*(4) |
| 14 " | 167 (7) | 8.98*(7) | 182 (4) | 11.47*(4) | 220 (4) | 10.44*(4) |

Purified $FeCl_3$.

| | | | | | | |
|---|---|---|---|---|---|---|
| At time of addition. | 95 (7) | 5.88 (7) | 98 (8) | 5.13 (8) | 107 (10) | 4.15 (10) |
| After addition. | | | | | | |
| 2 wks. | 99 (7) | 4.71 (7) | 92 (8) | 3.22 (7) | 108 (10) | 3.97 (10) |
| 4 " | 105 (7) | 4.94 (6) | 88 (5) | 2.74 (4) | 109 (7) | 4.62 (6) |
| 6 " | 106 (6) | 4.75 (6) | 87 (5) | 3.00 (5) | 121 (4) | 5.96 (4) |
| 8 " | 114 (5) | 6 00 (4) | 132†(1) | 2.00 (1) | 134 (4) | 5.53 (4) |
| 10 " | 131 (4) | 6.75 (4) | | | 139 (3) | 7.37 (3) |
| 12 " | 136 (3) | 6.31 (3) | | | 170 (1) | 11.04 (1) |
| 14 " | 138 (2) | 6.12 (2) | | | 188 (1) | 10.36 (1) |

Figures in parentheses refer to the number of animals used in computing the average values.

* Two figures included in each of these averages are for the week just prior to that shown in the table. This was occasioned by the fact that Hb determinations were made on one group of animals on the odd weeks instead of the even weeks.

† This animal just prior to death showed a pronounced edema of the neck and chest. At death, 4 days later, it weighed 105 gm.

The final dilution was such that 1 cc. usually contained 4 mg. of Fe. Water redistilled from glass containers was used throughout.

This specially purified ferric chloride was fed to five groups of anemic rats at levels of 2, 5, and 10 mg. of Fe per animal daily, 6 days per week. The same iron intake was fed to two other groups of anemic rats, but in the form of another preparation; namely, the ferric chloride solution which we first prepared and which had not been submitted to hydrogen sulfide treatment. We included these last animals as a further check because if the originally prepared ferric chloride had copper or other contaminants present, the higher levels of intake should show greater supplementing effects. These iron additions were made generally when the animals had been on the basal diet of whole milk for 5 weeks, at which time the hemoglobin was generally less than 6 gm. per 100 cc. of blood. The iron was added to the first feeding of milk in the morning and more milk was given as this was consumed. When care was exercised to keep the acidity of the iron solutions very low, no difficulty was encountered in getting good consumption of even the highest iron additions.

In Charts II and III we present in graphic form typical results obtained in these experiments and in Table I the data from all of this work are summarized. A study of these charts and the table shows that the ferric chloride which was not submitted to hydrogen sulfide precipitation was able to bring about marked increases in the hemoglobin levels, particularly of those animals receiving the higher dosage. All of the animals receiving this source of iron as an addition to their basal diet of whole milk increased in weight slowly throughout an experimental period of 14 weeks and maintained during most of that time a level of hemoglobin that was only slightly subnormal.

In contrast to this it may be seen that the animals receiving the *purified* ferric chloride did not respond with increased hemoglobin production and failed to increase in body weight. In Table I attention is called to the fact that the majority of the animals died in from 4 to 6 weeks following the iron addition to their diet. It must be noted, however, that two of the twenty-five animals that received the various levels of this purified iron solution did show some stimulation to hemoglobin regeneration. One, receiving 2 mg. of Fe daily, increased its titer from 4 gm. to 10 gm. of hemoglobin per 100 cc. of blood over a period of 10 weeks, with a slow increase in body weight during the same time. Follow-
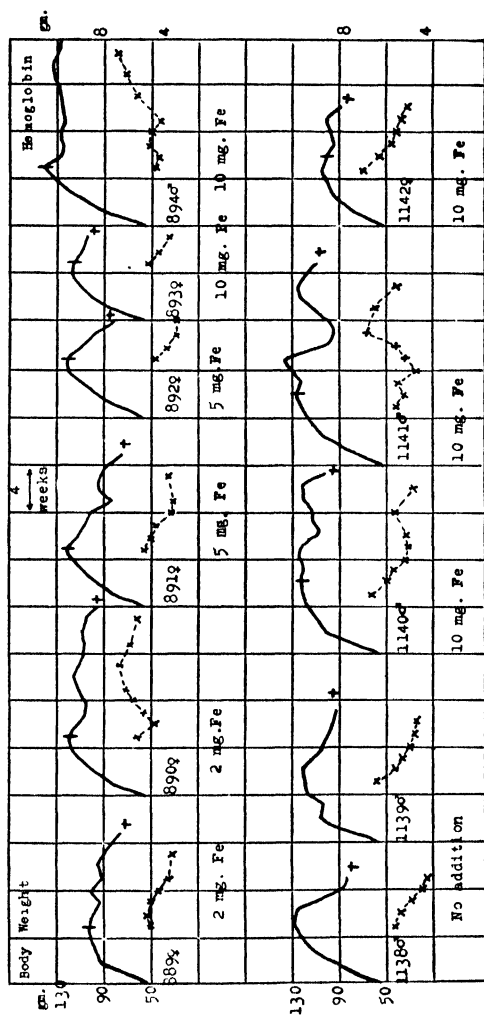
CHART III.   These two groups of rats received as a supplement to their basal diet of cow's whole milk the various levels of iron indicated from the *purified* solution of ferric chloride.

ing this, however, the hemoglobin and body weight fell off and the animal eventually died with a hemoglobin titer of 3 gm. per 100 cc. of blood.    The other rat, receiving 10 mg. of Fe daily, likewise gave high hemoglobin values ranging between 9 gm. and 11 gm. per 100 cc. of blood during the course of the experiment.    The effect of these figures on the average values may be noted in Table I.    It is impossible to explain the response of these individuals to iron feeding unless it is that fortuitous bodily reserves of other substances needed for the formation of hemoglobin, such as copper, were present in sufficient amounts.    All the other animals, however, reacted as we have indicated.    The results are shown graphically in Chart III.

### DISCUSSION.

The experiments discussed in this paper serve again to emphasize the importance of very small amounts of certain elements in the animal organism, and especially the importance of copper for hemoglobin formation.    It must be borne in mind that in the preparation of the original ferric chloride solution (1) only so called pure ingredients were used.    The iron wire was a Mallinckrodt product used for standardization.    However, as we have shown, even this source of iron contained sufficient copper to affect markedly the hematopoietic function in rats.    Analyses showing that copper is a contaminant of many iron salts have recently been published by Elvehjem and Lindow (3).

These results may serve in some measure to explain the many and conflicting reports in the literature with regard to the effect of iron in curing or preventing various experimental and clinical anemias.    It seems to us that the results of Beard and Myers (4), which have recently appeared, may be explained in this way. These workers found that an iron solution prepared from iron wire was effective at a level of 0.5 mg. of Fe daily, in curing rats made anemic on milk.    While our unpurified iron solution was ineffective at this level, it may be that the iron wire used by them was even more contaminated by copper than ours or that their animals had greater copper reserves.    It is suggested also that the matter of copper contamination may play a rôle in certain human anemias that respond to high iron dosage.    More particularly would this seem to hold in anemias of distinct nutritional origin, especially

where the diet is largely milk, such as is encountered in anemias of infancy.

### SUMMARY.

1. A pure iron salt, namely ferric chloride, when fed at very high levels did not serve to correct the anemia induced in young rats by a milk diet.

2. So called pure iron salts prepared from standard iron wire, when fed at sufficiently high levels, did cure this anemia unless they were freed from traces of copper; such as can be effected by treatment with hydrogen sulfide.

3. The part that copper may play in the cure of certain anemias, hitherto believed to have been effected by heavy dosage with iron salts, is discussed.

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# IRON IN NUTRITION.

## IX. FURTHER PROOF THAT THE ANEMIA PRODUCED ON DIETS OF WHOLE MILK AND IRON IS DUE TO A DEFICIENCY OF COPPER.*

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WITH THE COOPERATION OF EVELYN VAN DONK.†

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Last year it was shown (1) that the anemia developed in rats on a diet of cow's whole milk could be cured by the addition of the inorganic residues of various plant and animal materials. Later not only the ashes, but the acid extracts of the ashes were shown to be efficacious in this respect. Furthermore (2) a liver preparation made by Eli Lilly and Company under the auspices of the Committee on Pernicious Anemia of the Harvard Medical School was found to be potent in the presence of a sufficiency of iron when fed as such, as the ash, or as an acid extract of the ash. In addition, we were able to show that a fraction precipitated by hydrogen sulfide from the acid extract of the ash was effective, while other fractions were devoid of curative properties. This led us ultimately to the discovery that copper, hitherto not appreciated as an important element in animal nutrition, plays a very important rôle in certain processes of metabolism, namely, those concerned with hematopoiesis.

As a result of this discovery we were able to explain the curative action of the various preparations and materials which we had found active. We also proved by analysis that copper in varying amounts was present in our potent fractions. However, there

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

·† E. R. Squibb and Sons Fellow.

immediately arose the question as to whether or not copper was the only element occurring in our preparations which could supplement iron in the cure and prevention of the particular type of anemia which we were studying.   In this paper and others to follow, we wish to present the results of our past year's experiments bearing on the specificity of copper as a supplement to iron in the cure of nutritional anemia.

<div align="center">EXPERIMENTAL.</div>

In our previous paper we showed that the fraction precipitated by hydrogen sulfide from an acid extract of the ash of the Lilly preparation[1] was effective in bringing about increased hemoglobin formation while the other fractions were impotent.   To determine whether there were other active elements present in this fraction, in addition to copper, we undertook among other things to separate the copper from the other elements present.   This we accomplished by electrolytic deposition of the copper by the following procedure.

12 gm. of the Lilly preparation were ashed and the copperarsenic group precipitated from a HCl extract of the ash in the usual manner by $H_2S$.   The precipitate was dissolved in nitric acid, and the solution evaporated almost to dryness to remove the free nitric acid.   The nitrates were taken up in water and the solution made to a volume of 5 cc.   The copper was deposited on a platinum gauze electrode by placing the solution in the apparatus described by Pregl (3).   The electrolysis was also carried out according to his method except that it was repeated five times to insure complete removal of all the copper.   After each deposition the cathode was immersed in nitric acid and washed to remove the copper from the electrode.   The nitric acid  solution was evaporated to dryness, taken up in 1 cc. of HCl, and diluted to 40 cc.   The residue was also made up to 40 cc.

The electrolyzed copper and the residue from the electrolysis of the hydrogen sulfide fraction were then fed to anemic rats, being added to a basal diet of whole milk and iron.[2]   Both of these

[1] Eli Lilly and Company preparation,  Lot W373.
[2] The basal diet was cow's whole milk fed *ad libitum* plus 0.5 mg. of Fe as ferric chloride solution 6 days per week.   During the period (5 weeks generally) in which the animals were made anemic, whole milk only was fed.

substances were fed at a level representing 0.3 gm. of the original
Lilly preparation (copper content of 0.3 gm. = 0.05 mg.).   Be-
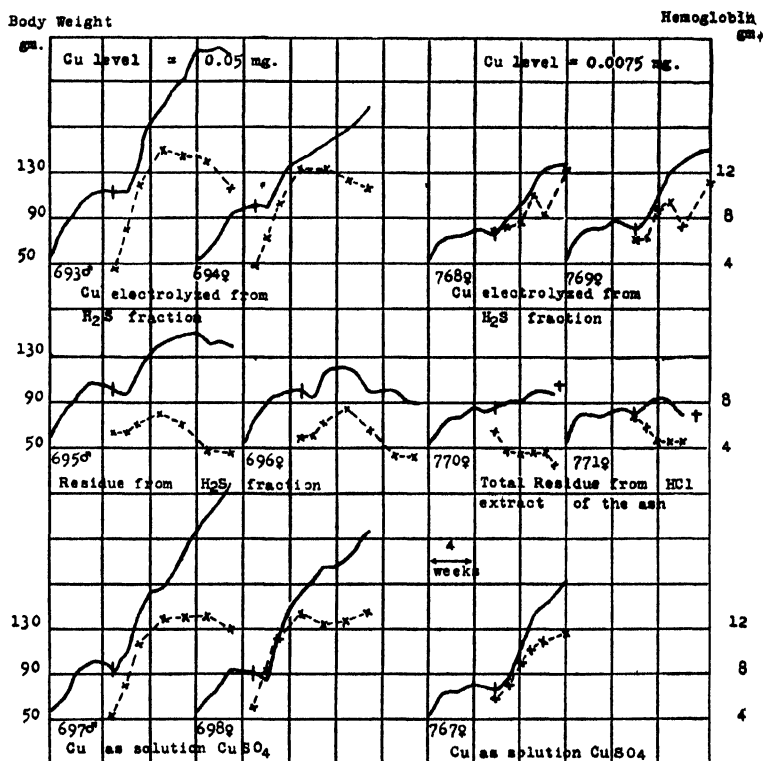


CHART I.   In this chart are presented data showing that the removal of
copper from the acid extracts of the ashes of the Lilly preparations leaves
the residues inert.   Copper introduced as a solution of the sulfate is as po-
tent as that electrolyzed from the extract.   In this and the following charts
the solid line represents body weight; the broken line represents hemoglobin
in gm. per 100 cc. of blood.   The line across the curve of body weight marks
the addition to the basal diet, the dagger indicates death.

sides these substances we also fed 0.05 mg. of Cu as a solution of
pure copper sulfate.   The results secured from adding these

The iron and other additions were mixed in the first morning feeding and
more milk given as this was consumed.   All the additions were also made
6 days per week.

three materials are shown graphically in Chart I (Rats 693 to 698). It is to be noted that both the copper electrolyzed from the H₂S fraction and that from copper sulfate brought about prompt and marked increases in the hemoglobin level and body weight, while the residue remaining from the copper electrolysis was quite inactive.

In a further experiment of the same nature the copper was again electrolyzed from the hydrogen sulfide fraction but the residue instead of being fed as such was added to the filtrate from the H₂S precipitate and this residue, representing the total residue from the acid extract of the ash, was fed. As before, copper from a copper sulfate solution was included in the group. In this experiment the electrolyzed copper and the residue were again fed at levels representing 0.3 gm. of the preparation. This, however, was a new sample of liver preparation which we had received from Eli Lilly and Company (designated by them No. 343) and since it contained less than one-sixth the copper content of the old sample, the copper level was only 0.0075 mg. of Cu daily.

The results of this experiment, also shown in Chart I (Rats 767–771) were of exactly the same order as already described. They show convincingly that the only material present in the HCl extract of the ash of the liver preparations put out by Eli Lilly and Company was copper and that copper obtained from a pure copper salt ($CuSO_4 \cdot 5H_2O$) was equally as efficacious as that electrolyzed from the extract of the ash.

*Further Experiments on Dried Liver, Liver Preparations, $H_2S$ Fractions of Lilly Preparations, and Copper.*

Concurrently with the above experiments we also carried out others in which the Lilly preparation (No. 373 or 343) a H₂S fraction of the acid extract of the ash of the Lilly preparation, and copper as pure copper sulfate were compared as to their ability to supplement the basal diet of milk and iron. The three additions were made in quantities such that they introduced equal amounts of copper, the copper intakes ranging from 0.001 to 0.05 mg. of Cu. In other experiments of the same nature we included several liver preparations which were likewise fed in such amounts that they introduced equivalent amounts of copper. These liver preparations included dried beef liver which we had

prepared ourselves, two liver preparations put out by the Wilson Laboratories of Chicago (which we have designated Wilson I and Wilson 9444), and two Lilly preparations.  These different products represented liver tissue prepared in a variety of ways and of varying degrees of refinement.  The copper content of each varied accordingly.  As an example it may be mentioned that 27 mg. of Wilson I contained 0.01 mg. of Cu, while Wilson 9444 contained so much less Cu that it required 166 mg. of it to introduce the same amount.

We reasoned that by using a variety of preparations and comparing them all on the basis of their copper content, any other substance (or substances), *organic* or *inorganic* in nature, that was potent in hemoglobin regeneration would reveal its presence. This would follow especially when the copper intake was very low.

Fifteen litters of rats, representing a total of 80 animals, were used for these studies.  Typical results are presented in Charts II to V.  Although we present only a few of the many records we possess, we can state that the results were very uniform.  All the additions were remarkably uniform in their potency.  This was as true of the copper added by means of a copper sulfate solution as that introduced by any of the other preparations.  The hydrogen sulfide fractions of the acid extracts of the ashes of the Lilly preparations were as potent as the preparations themselves, while an equivalent amount of copper as $CuSO_4 \cdot 5H_2O$ was equally effective.

In only one series, that in which the copper intake was the lowest, namely 0.001 mg. of Cu, was any irregularity found.  Here it was apparent that the dosage was too low to bring about complete regeneration in all cases.  Some individuals with apparently good reserves made slight gains in hemoglobin levels and increased their body weight, while others failed to respond and died after varying periods.  At all other levels of intake the results were as we have indicated.

It is unnecessary to discuss at any great length the results which we have presented and we do not propose to do so at this time. In a later publication we shall comment on these data and discuss them in the light of data still to be presented.  It would seem that the following summary is justified.
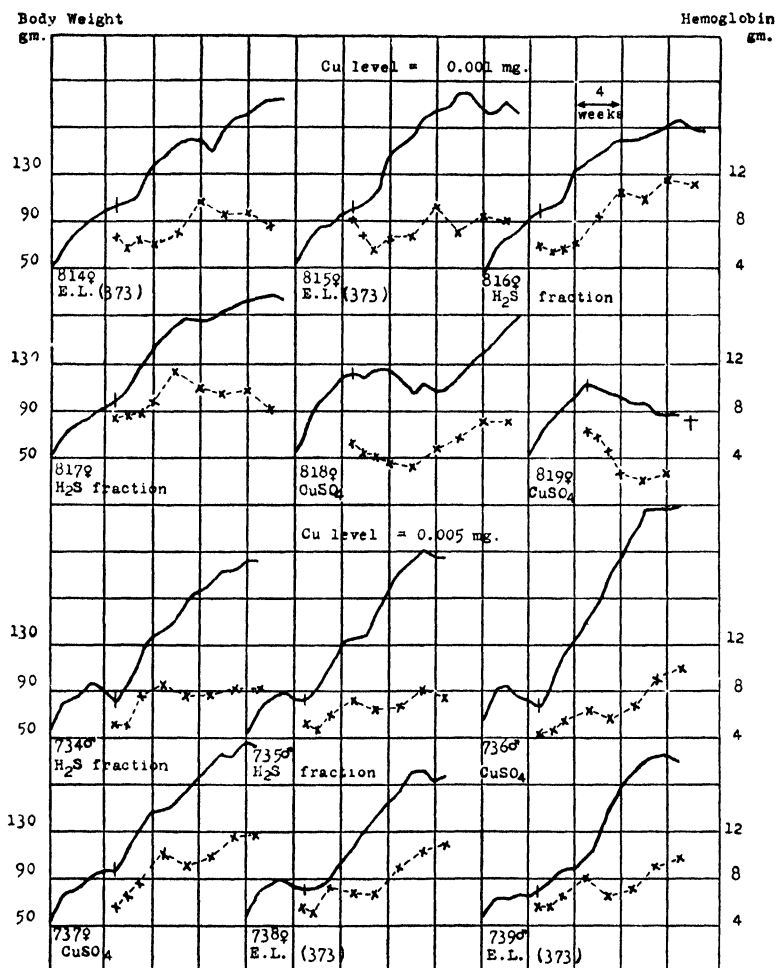
CHART II. Here are presented the results secured when there were added to the basal diet of milk and iron the Lilly preparation (No. 373), the hydrogen sulfide fraction of the HCl extract of the ash of this preparation, or copper as a solution of copper sulfate. Two levels of copper intake are shown.
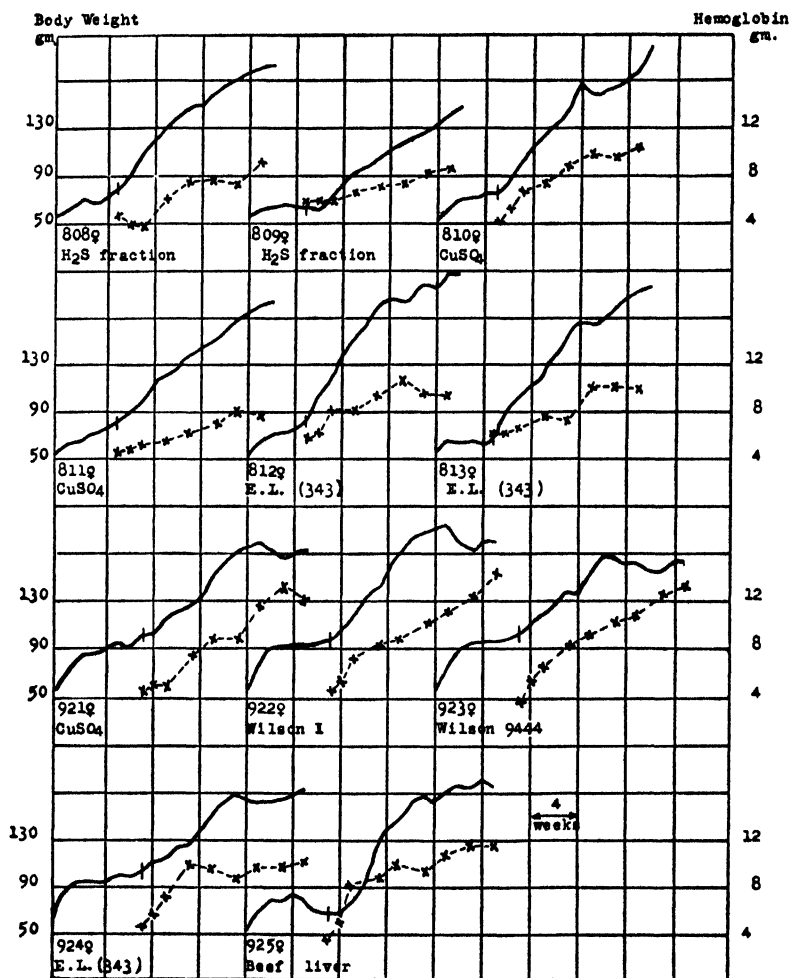
CHART III. All the additions indicated above introduced the same amount of copper; namely, 0.0025 mg. of Cu daily six times per week.
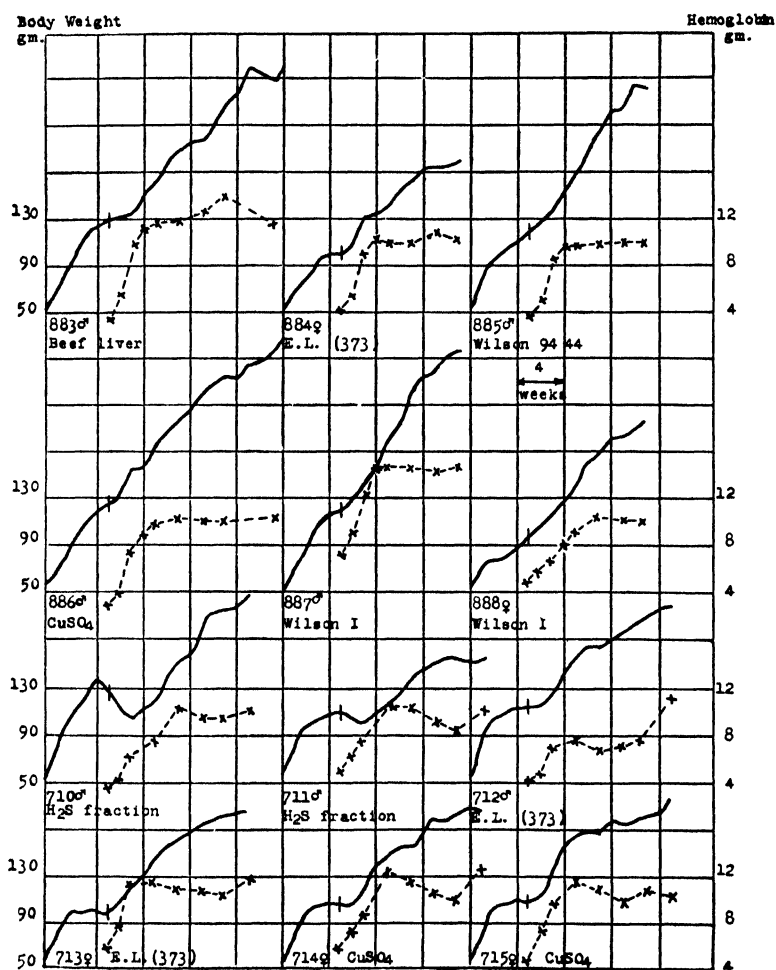
CHART IV.   The above additions to the basal diet of milk and iron were fed in such amounts that they all introduced 0.01 mg. of Cu daily six times per week.
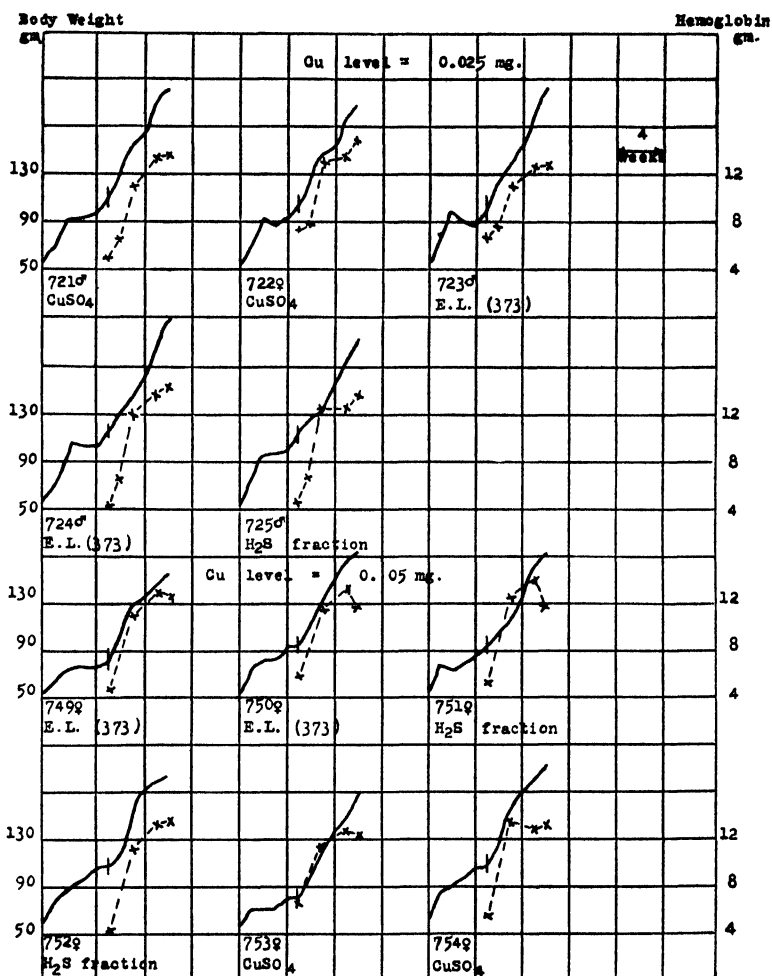
CHART V. Here are shown the results secured when the above additions were made to the basal diet of milk and iron in amounts sufficient to introduce either 0.025 or 0.05 mg. of Cu daily, six times per week. As in the previous charts, it may be noted that all the additions serve to bring about hemoglobin regeneration.

### SUMMARY.

The supplementing of a basal diet of whole milk and iron with several liver preparations, with $H_2S$ fractions of the acid extracts of the ashes of two of them, and with copper as a solution of copper sulfate, all on the same levels of copper intake, has shown that all serve equally well to cure the nutritional anemia produced by the basal diet. This is additional and convincing proof that the deficiency of this basal diet is *inorganic* in nature and that this inorganic deficiency is copper only.

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# ON THE PRESENCE OF ALUMINUM IN PLANT AND ANIMAL MATTER.

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PLATE 1.

In an article entitled, "A Study of the Possible Rôle of Aluminum Compounds in Animal and Plant Physiology," by McCollum, Rask, and Becker,[1] these authors arrived at the conclusion that, "Aluminum is not a constituent of either plant or animal matter." Since this conclusion is so contrary to what has been found by all previous investigators, we have performed the following experiments in order to ascertain whether plant and animal matter does not contain aluminum in quantities sufficient to be detected by means of the spectroscope.

The instrument used was a Krüss quartz prism spectrograph and the materials examined were whole hen's egg exclusive of shell, potato, carrot, English walnut meat, peach pit, apricot pit, pop-corn, Lima bean, navy bean, lupin bean, peanut kernel, lean beef, beef tendon, and human cancerous tissue excised by the surgeon from the breast of a woman of middle age. In all of these cases aluminum was found to be present in quite sufficient amounts to be detected with certainty with the spectroscope.

Extreme precautions were used to guard against all possible contamination of the materials while they were being cleansed, ashed, and finally tested spectroscopically. Adhering dirt was removed by careful washing with distilled water. The materials were then dried and ashed in platinum dishes at a dull red heat. No reagents whatever were used, and so the only possible source of contamination of the material with aluminum was through the atmosphere. Consequently, while the work was in progress, the air of the laboratory was kept as dust-free as possible. The

[1] McCollum, E. V., Rask, O. S., and Becker, J. E., *J. Biol. Chem.*, **77,** 753 (1928).

platinum dishes were cleansed with special care before each deter-
mination.   Some of them were new, that is they had never before
been used for other determinations, so that contamination from
the material of the dishes is extremely unlikely.   McCollum,
Rask, and Becker ashed in silica dishes.   We tried this and found
that silica dishes are strongly attacked by the alkalies present,
even when the ashing is conducted at low temperatures.   This
method can consequently not be recommended.   It should be
definitely stated, however, that even when silica dishes were used
the ash when spectroscopically tested showed aluminum to
be present.

An electric arc was formed between small rods of electrolytic
copper, the lower one being cup-shaped so as to hold the ash to
be examined.   Examination in the electric arc was found to be
the most satisfactory method, though the electric spark method
was also employed.   The electrodes were about 1 cm. apart, and in
producing the arc a direct current of 5 amperes under a pressure of
110 volts was used.   In producing the spark spectrum a transformer
giving 20,000 volts was employed, a condenser of 0.005 micro-
farads capacity was shunted across the transformer, and a react-
ance was connected in series with one of the electrodes to diminish
the intensity of the lines due to the elements of the atmosphere.
Photographs of the spectra were made on x-ray films with the
Krüss quartz spectrograph.   In each case the spectrum of the
copper alone was photographed simultaneously with that of the
ash.   This was accomplished by means of a diaphragm which
separated the field into three portions, the upper and lower ones
giving the spectrum of copper alone, while the middle one gave
that of copper plus ash.

It is well known that the spectrum of an element contains lines
of varying degrees of intensity and persistence, as the substance
tested is diluted with foreign inert matter.   The more intense
lines are not necessarily the more persistent ones.   According to
the work of de Gramont[2] the two lines of wave-lengths 3961.5 and
3944.0 Ångström units are the most sensitive or persistent lines of
aluminum; that is to say, the smallest detectable amounts of
aluminum will show these two lines.

[2] de Gramont, A., Wave length tables, London (1923).

A large number of examinations were made, mainly by means of the arc, for this proved to be far better than the spark. When the spark is employed the ash is so rapidly blown out of the electrode cup that the material is exposed only momentarily to the exciting action and so the detection of the aluminum is readily missed. An effort was made to fuse the ash in the electrode cup before subjecting it to the spark. However, it was found that by thus fusing the ash in the arc (although the operation required but a few seconds) the aluminum was volatilized to such an extent that it could no longer be detected in the spark spectrum. The use of the arc as described above obviated these difficulties which are met when the spark is employed. In all cases aluminum was found to be present with certainty. No effort was made to determine it quantitatively; but it was present in quite small amounts for only the most sensitive lines mentioned above were found with any degree of distinctness.

While a large number of spectra were photographed, it seems quite unnecessary to reproduce all of these here, particularly since they all show the presence of aluminum; in Figs. 2 to 8 are presented seven typical spectra. Fig. 1 gives the spectrum of copper alone. In this the lines corresponding to 3000, 3500, 4000, 4500, and 5000 Å. respectively are correspondingly designated for purposes of orientation. In Figs. 2 to 8, inclusive, are given the spectra of the ash of the various materials. The middle band of each figure is the spectrum of the ash plus copper, while the upper and lower bands are due to the copper only. That the latter was free from aluminum is clearly apparent, also that aluminum is present in each ash tested is quite evident for in each case the characteristic lines corresponding to 3961.5 and 3944.0 Å. can be seen. In the negatives themselves these lines are much clearer than in the accompanying figures, for repeated reproduction dims them considerably. The spectra of Figs. 2 to 6 were got by means of the arc; those of Figs. 7 and 8 by means of the spark. Fig. 2 represents the ash of potato; Fig. 3 that of carrot; Fig. 4 that of English walnut meat; Fig. 5 that of cancerous human breast; Fig. 6 that of peach pits; Fig. 7 that of English walnut meat (by spark); and Fig. 8 that of carrot (by spark).

In the light of the facts here presented it is clear that aluminum is actually present in plant and animal matter and that the conclu-

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sion of McCollum, Rask, and Becker that "aluminum is not a constituent of either plant or animal matter" is founded upon error and cannot be maintained.

Our thanks are due to Mr. Albert C. Krueger for help in carrying out some of the experiments.

EXPLANATION OF PLATE 1.

FIG. 1. Arc spectrum of copper electrodes.

FIG. 2. The middle band represents the arc spectrum of potato ash. The upper and lower bands are copper alone    Note that the copper is free from aluminum.    Characteristic aluminum lines are located at 3961.5 and 3944.0 Å.

FIG. 3. The middle band represents the arc spectrum of carrot ash.

FIG. 4. The middle band represents the arc spectrum of the ash of English walnut meat.

FIG. 5. The middle band represents the arc spectrum of the ash of cancerous human breast.

FIG. 6. The middle band represents the arc spectrum of the ash of peach pits.

FIG. 7. The middle band represents the spark spectrum of the ash of English walnut meat.

FIG. 8. The middle band represents the spark spectrum of carrot ash.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

FIG. 8.

(Kahlenberg and Closs: Aluminum in plant and animal matter.)

# THE EXCRETION OF LEAD IN URINE.*

By HORACE MILLET.

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The excretion of lead in urine has recently been studied by several investigators. Aub and his associates (Aub, Fairhall, Minot, and Reznikoff, 1926) have observed the effect of medication on the excretion of lead in lead-poisoned patients. Kehoe and others (Kehoe, Edgar, Thamann, and Sanders, 1926) have measured the excretion of lead from normal persons. Both investigations show that the major part of the excretion is found in the feces. Kehoe, whose method seems to have been the more sensitive, noted that healthy men, many of whom had had no definite exposure to lead, excreted lead normally in urine and feces. This author was led to question whether any source of lead less widely distributed than food could occasion a fairly uniform excretion of lead in men of varied occupations and modes of life.

The present investigation deals with the determination of the total lead excreted in the urine of (*a*) a few healthy persons, (*b*) a number of cancer patients who had received intravenous injections of colloidal lead phosphate, and (*c*) cancer patients resident in the same nursing home, who had not been treated with lead. In the case of the cancer patients, the mixed 24 hour urine was taken for estimation.

Lead has been estimated as lead ion by the use of a fluid lead amalgam electrode, operated in the absence of oxygen, which has recently been described by the author (Millet, 1929).

The treatment of the urine has been based on the work of Fairhall (1924), who found that ammonia precipitates practically all

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the lead from urine.   An amount of urine varying from 100 to 200 cc. was treated with 10 to 20 cc. of concentrated ammonia and allowed to stand overnight.   The resulting precipitate was filtered through a Whatman No. 1 filter paper and washed once or twice with distilled water.   The precipitate and filter paper were then ashed by gentle heating in a silica crucible, excess heating being avoided and the ashing being incomplete.   The contents of the crucible were subsequently extracted with about 10 cc. of 0.5 N hydrochloric acid in two successive portions, the extraction taking about 30 minutes.   The extract was made up with water to a volume of 50 cc. and a portion titrated to estimate the strength of the acid, which varies between 0.05 and 0.10 N.

The lead content of the acid solution was determined by means of the lead electrode already mentioned, the E.M.F. being reproducible to 0.3 millivolts.   Lead ion activities have been converted to lead ion concentrations by the formula deduced by the author (Millet, 1929), the ionic strength being taken as the strength of the acid.   The electrometric method is accurate to about 2 per cent, but owing to the risk of contamination with lead in the treatment of the urine the accuracy of the estimations may be lessened.

The results obtained are collected in Table I.   It is evident that, as found by Kehoe, cancer patients not specially exposed to lead have a normal excretion which is variable but averages 0.08 mg. per liter.   It is interesting to note that the average figure found is the same as that recorded for healthy persons by Kehoe, whose method was less accurate.

The estimations on the urines of cancer patients treated with lead have been grouped according to the number of days which has elapsed since the last injection.   In the case of one or two patients in the last group the lead had been given in the form of a metal-metal oxide colloid.   It will be seen that there is no evidence that the lead injected is at any time being excreted in the urine. Within the limits of variation that would reasonably be anticipated, the mean excretion in each group of cancer patients is constant, whether stated in mg. per liter or in mg. per day.

Since single doses of the lead phosphate colloid contained usually 50 mg. of lead, an increase above the normal level of excretion would be expected to appear if the lead injected were in fact being excreted through the kidney.   It may be mentioned that lead

TABLE I.

*Excretion of Lead in Urine.*

| Group. | Case No. | Days since last injection. | Lead in mg. per liter urine. | Lead in mg. per day. |
|---|---|---|---|---|
| Healthy persons | 1 | | 0.050 | |
| | 2 | | 0.053 | |
| | 3 | | 0.044 | |
| | 4 | | 0.076 | |
| Gynecological cases. | 1 | | 0.039 | |
| | 2 | | 0.164 | |
| Cancer patients, no lead injected. | III | . | 0.047 | 0.083 |
| | X | | 0.151 | 0.107 |
| | XVII | | 0.048 | 0.027 |
| | XVIII | | 0.162 | 0.161 |
| | XXII | | 0.052 | 0.044 |
| | XXIII | | 0.088 | 0.080 |
| | XXIV | | 0.027 | |
| | XXIV | | 0.068 | |
| | XXV | | 0.125 | 0.163 |
| | XXVII | | 0.045 | 0.024 |
| | XXVIII | | 0.077 | 0.092 |
| Average   . . . . . . . . . . . . .   . . . | | | 0.081 | 0.087 |
| Cancer patients, lead injected 0 to 26 days previously | II | 0 | 0.063 | 0.075 |
| | VI | 0 | 0.100 | 0.085 |
| | VIII | 0 | 0.061 | 0.076 |
| | III | 1 | 0.039 | 0.044 |
| | I | 1 | 0.149 | 0.246 |
| | II | 12 | 0.048 | 0.067 |
| | IV | 17 | 0.047 | 0.040 |
| | XII | 24 | 0.102 | 0.122 |
| | VI | 26 | 0.088 | 0.088 |
| Average..   . . . . . . . . . . . . .   . . . . . | | | 0.077 | 0.094 |
| Cancer patients, lead injected 28 to 67 days previously. | II | 28 | 0.083 | 0.071 |
| | III | 28 | 0.055 | 0.063 |
| | VIII | 28 | 0.068 | 0.054 |
| | II | 30 | 0.020 | 0.018 |
| | XIV | 32 | 0.050 | 0.057 |
| | IV | 33 | 0.175 | 0.099 |
| | II | 41 | 0.147 | 0.171 |
| | VI | 42 | 0.069 | 0.043 |
| | XIV | 43 | 0.042 | 0.054 |
| | V | 46 | 0.073 | 0.062 |
| | V | 67 | 0.073 | 0.079 |
| Average..  ..  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . | | | 0.078 | 0.070 |

TABLE I—*Concluded.*

| Group. | Case No. | Days since last injection. | Lead in mg. per liter urine. | Lead in mg. per day. |
|---|---|---|---|---|
| Cancer patients, lead | VIII | 141 | 0.125 | 0.160 |
| injected 141 to 801 | IX | 142 | 0.186 | 0.186 |
| days previously. | XIII | 152 | 0.046 | 0.072 |
| | XIX | 187 | 0.084 | 0.095 |
| | XX | 198 | 0.037 | 0.044 |
| | XVI | 328 | 0.053 | 0.072 |
| | XXVI | 672 | 0.057 | 0.065 |
| | VII | 801 | 0.302 | 0.197 |
| Average. ..................... | | | 0.111 | 0.111 |

phosphate, which is very insoluble, produces practically no toxic symptoms and damages the kidney very little.

The lead injections have been made in Liverpool under the direction of Professor W. Blair Bell, to whom the author is indebted for the material and particulars of the cases.

SUMMARY.

Estimations have been made by an electrometric method of the excretion of lead in urine.

In cancer patients who had received injections of a lead phosphate colloid and in cancer patients not treated with lead the excretion was about the same, averaging about 0.085 mg. of lead per liter.   There is a definite normal excretion of lead in urine.

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# SPARING ACTION OF FAT ON THE ANTINEURITIC VITAMIN B.*†

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About 3 years ago, in an attempt to use more rigorously purified diets than had hitherto been employed in nutrition research, this laboratory began to employ dietary mixtures consisting essentially of casein and sucrose with the added salts and vitamins. Our experiments yielded some surprises which led us to wonder whether a new dietary factor was essential for life. Prominent in the phenomena brought to light was the favorable effect secured by the addition of fats to the diet. It was true that other substances (liver, lettuce, egg yolk) also conferred this improvement, substances which had in common the possession of considerable amounts of the antineuritic vitamin B. It was, in fact, possible to show that the favorable effect of these other substances was in proportion to their content in antineuritic vitamin B. Thus, though we thought we had employed an adequate amount of vitamin B,[1] this experience finally led us to the conviction that deficiency in the antineuritic vitamin B was an actual and main trouble with the sucrose-casein diet. This was confirmed by the

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* Due to the confusion regarding the nomenclature of the water-soluble B vitamins, we have carefully refrained from the sole use of letters. For the heat-labile factor of yeast, we have kept the original nomenclature, the antineuritic vitamin B. For the heat-stable factor of yeast often referred to by various workers as P-P, F, and G, we have employed the term heat-stable water-soluble vitamin.

† Aided by grants from the Committee for Research on Problems of Sex of the National Research Council, and from the United States Bureau of Dairying, and the School of Agriculture and Board of Research of this institution.

[1] 700 mg. daily of a whole dried bakers' yeast (Fleischmann) had been employed and an increase to 1 gm. daily was without appreciable effect, so that we were led off the "scent" of a vitamin B deficiency.

TABLE I

*Composition of Diets Employed in This Study.*

| Diet No | No fat | | | 10 per cent fat | | | | | | 50 per cent fat | 51 per cent fat | | | 54 per cent fat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 540 | 542 | 414 | 550 | 415 | 563 | 564 | 565 | 551 | 559 | 571 | 572 | 573 | 579 |
| Casein (L-3)* | 25 0 | 20 0 | 19 0 | 20 0 | 20 0 | 23 0 | 23 0 | 23 0 | 27 0 | 36 0 | 38 0 | 38 0 | 38 0 | 40.0 |
| Autoclaved yeast.† | | 10 0 | 10 0 | 10 0 | 10 0 | 10 0 | 10 0 | 10 0 | | 10 0 | 10 0 | 10 0 | 10 0 | |
| Sugar (commercial) | 75 0 | 70 0 | | 59 0 | | 56 0 | 56 0 | 56 0 | 59 0 | | | | | |
| Corn-starch (dextrinized)‡ | | | 67.0 | | 59 0 | | | | | | | | | |
| Salts 185.§ | 4.0 | 4.0 | 4 0 | 4 0 | 4 0 | 4 0 | 4 0 | 4.0 | 4 0 | 4 0 | 4 0 | 4 0 | 4 0 | 4.0 |
| Lard | | | | 10 0 | 10 0 | | | | 10.0 | 50 0 | | | | 53.0 |
| Crisco.‖ | | | | | | 10 0 | | | | | | | | |
| Wesson oil.¶ | | | | | | | 10 0 | | | | 55 0 | | | |
| "Synthetic" Wesson oil | | | | | | | | 10 0 | | | | 55 0 | 55 0 | |

All diets were supplemented with 2 drops of cod liver oil (Patch) daily.

* *Casein (L-3)*.—Commercial casein (Golden State Milk Products Company), which had been precipitated from milk with hydrochloric acid, dried, and delivered to us in sacks, was extracted in wooden tubs with acidulated water (40 cc. of glacial acetic acid to 40 liters of filtered tap water). The acidulated water was siphoned off and replaced twice daily for 6 days. On the 7th day the casein was washed twice with distilled water replacing the acidulated tap water. The water was thoroughly drained from the casein and the casein washed once with 50 to 80 per cent alcohol and once with 85 to 95 per cent alcohol followed by one ether treatment. It was then spread in pans and the ether allowed to dissipate at room temperature under a fan.

† *Autoclaved Yeast*.—Whole dried yeast (bakers') was generously supplied us by the Fleischmann Company of New York. It was spread in pans to a depth of less than 1 inch and autoclaved for 5 hours at 18 to 20 pounds pressure. This yeast was added as the source of the thermostable water-soluble factor throughout the experiment. Abundant trials showed it to be almost entirely, if not absolutely, free from antineuritic vitamin B. A whole, dried brewers' yeast of high potency was used as the source of the thermolabile or antineuritic factor B. It was generously furnished by the Vitamin Food Company through Dr. Edward A. Rumley.

‡ Dextrinized corn-starch was prepared by pouring cold starch paste into boiling water, boiling for 5 minutes, spreading in thin layers in aluminum pans, and drying at 110–120°.

§ *Salt Mixture 185, after McCollum.*—Sodium chloride 51.0, crystals of magnesium sulfate 159.6, monobasic sodium phosphate 104.1, monobasic calcium phosphate 162.0, dibasic potassium phosphate 286.2, ferric citrate 35.4, and calcium lactate 390.0.

‖ Crisco, the well known name for a partially hydrogenated cottonseed oil. It is always hydrogenated to a melting point of about 38°.

¶ Wesson oil, the trade name for the cottonseed oil marketed for table use. It is a "winterized" oil, which has been chilled to remove the "stearine." The product is marketed by the Southern Cotton Oil Company, and represents a carefully standardized product which has been thoroughly refined and purified.

improvement invariably secured by the administration of all concentrated sources of antineuritic vitamin B such as wheat germ, brewers' yeast, rice polish extract, etc.

A novel problem is presented by the benefit due to fats, substances not hitherto thought to be seriously contaminated with the water-soluble vitamins. The preliminary data convinced us that fat was in some obscure way related to the body's needs in the antineuritic vitamin, and a note[2] was published to the effect that the presence of fats always decreased the amount of antineuritic vitamin B necessary for any definite amount of growth secured with a fat-free diet. We ventured to term this phenomenon "the sparing action of fat on the antineuritic vitamin."

### Methods.

A systematic effort to study this phenomenon was now made. Litters of female rats 21 days old were selected and sisters distributed as far as possible to each of several dietary groups. All diets used carried the heat-stable, water-soluble factor free from antineuritic vitamin B, the heat-labile factor, as autoclaved yeast, so that we were working with only one factor as a variable. Throughout the course of the experiment the animals were maintained in groups of three in cages with wire mesh floors. The feeding boxes for administering supplementary food (yeast doses, etc.) were also equipped with wire mesh floors. The animals were watched daily for breakdown of the vaginal closing membrane and thereafter for estrous changes in the vaginal smear indicative of ovulation. (The small circles interrupting the growth curves in our charts indicate times of ovulation.) The diets employed are given in Table I. Special effort was made to keep the nutritive ratio approximately 1:3 so that even on the highest fat diets animals could not suffer from protein starvation.

*Growth on Sucrose-Casein Diet and Improvement Due to Coincident Presence of Fat with Any Level of Antineuritic Vitamin B.*

Fig. 1 shows the influence of three different fats added to the basal diet at levels of 10 and 50 per cent *in the absence of antineuritic vitamin B.* There is very little added growth due to

[2] Evans, H. M., and Lepkovsky, S., *Science,* **68,** 298 (1928).

10 per cent fat, but at the 50 per cent level of fat a very considerable growth takes place. *We may note that with 50 per cent of lard, animals have attained a weight of 120 to 160 gm. and are still alive and in fairly good condition at the end of 6 months.* With 51 per cent Crisco and Wesson oil results are not so uniform, but two animals in each group are still alive after being on the diet for $4\frac{1}{2}$ months. The growth in these groups is rather irregular, one animal in each group attaining a weight well over 100 gm. and another attaining a weight of close to 100 gm.· One animal in each group died soon after the initiation of the experiment.

Fig. 2 represents the same oils at the same levels, the only difference being that 50 mg. of brewers' yeast were added as a low source of antineuritic vitamin B. In this series the increment of growth at the level of 10 per cent of fat is very evident over that shown by the animals in the group receiving no fat. It will be recalled from Fig. 1 that 10 per cent of fat made very little difference in the absence of antineuritic vitamin B, but it makes a considerable difference in the presence of 50 mg. of brewers' yeast. These animals are now 5 months of age and only recently have begun to decline in weight. At the high level of fat (50 to 51 per cent) the improvement is very much beyond that due to 10 per cent of fat in the case of all fats studied; these animals have only recently reached a plateau of growth at approximately 160 gm. of body weight.

Fig. 3 presents the performance at the same levels of fat with Crisco and Wesson oil when 200 mg. and 800 mg. respectively of brewers' yeast are added to supply the antineuritic vitamin B. In these cases it is seen that the maximum improvement due to fat is shown at the 10 per cent level.[3] The difference between the 200 and 800 mg. levels of yeast is shown only·in the complete absence of fat. The data presented may be briefly summarized as follows: In the absence of antineuritic vitamin B it takes relatively more fat to bring into evidence the sparing action of fat.

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[3] For some unknown reason the 50 per cent Wesson oil actually brings about a poorer performance than 10 per cent Wesson oil. We suspect that the ingestion of such a large amount of the oil exerted a deleterious effect, for there was also a slight diarrhea, which persisted during the 1st month of the experiment. There is no significant difference between the high and lower levels of Crisco

Little sparing action is evident when 10 per cent of fat is added but with 50 per cent of fat the sparing action is marked. At the low level of antineuritic vitamin B, a marked effect of 10 per cent of fat is evident, further marked improvement taking place at its high level; whereas at the higher levels of yeast little (10 per cent) fat brings about a maximum effect.

Fig. 4 shows that fat acts in its capacity as a sparer of antineuritic vitamin B when dextrinized corn-starch is used as a source of energy in place of sucrose. In this figure it is clearly seen that superior growth is obtained when starch replaces sugar, but that if a portion of the starch is replaced with 10 per cent of fat there is further added growth. This is evident in the presence of 50 mg. of brewers' yeast as well as in the entire absence of yeast.

### *Is Fat Contaminated with the Antineuritic Vitamin B?*

The question, of course, arises as to whether the sparing action of fat is due to the fat *per se* or to antineuritic vitamin B with which the fat may be contaminated. Attempts to extract antineuritic vitamin B from one of these fats were without success.

Wesson oil was dissolved in 5 times its volume of ether and shaken with 4 times its volume of 25 per cent alcohol containing 1 cc. of concentrated sulfuric acid per liter. This was repeated three times. The 25 per cent alcoholic extract was concentrated in a vacuum and neutralized and fed at a level equivalent to 5 gm. of the original fat daily to animals receiving sufficient of the thermostable water-soluble vitamin as autoclaved yeast, but only enough antineuritic vitamin B so that they plateaued at about 90 to 95 gm. in weight. No response was obtained.

Abundant experience in this laboratory under the same conditions has convinced us that should the extracts have contained any antineuritic vitamin B, an immediate response would have been obtained.

Another attempt was made by dissolving 500 cc. of Wesson oil in glacial acetic acid with enough ether added to bring about solution of the Wesson oil. The solution was thoroughly shaken with fullers' earth, filtered, and the earth fed to animals receiving a diet low in antineuritic vitamin B as the only limiting factor without results.

To the above filtered solution, water was carefully added until

the Wesson oil separated out. This fat-free acetic acid solution was shaken with fullers' earth, filtered, and the earth fed to animals on a diet low in antineuritic vitamin B without response.

Thus we were unable to shake out antineuritic vitamin B either from the glacial acetic acid solution containing the fat or from the solution after the fat had been separated out.

A third attempt was made when 500 cc. of Wesson oil were dissolved in a mixture of alcohol and ether and shaken with clay. The clay was fed without results.

This attempt was repeated with lard. The lard was dissolved in about 6 times its volume of ether and thoroughly shaken out five times with water containing 1 cc. of glacial acetic acid per liter. The acidulated water was concentrated in a vacuum and mixed with our Diet 542 so that 1 gm. of the diet would carry extract equivalent to 1 gm. of lard. The residual lard was incorporated to form Diet 559, so that 1 gm. of diet would carry only 0.5 gm. of lard. Very little or no response was obtained with the extract, while the extracted fat caused an immediate and marked response in animals receiving a diet low in antineuritic vitamin B. Since our Diet 542 plus the lard extract carried extract equivalent to 100 per cent of lard, and since it brought about no marked response (while only one-half the concentration of the extracted lard brought about an immediate and marked response) it must be admitted that lard carries little if any antineuritic vitamin B as an impurity.

A more rigorous treatment of the oil was now instituted, a saponification, distillation, and reesterification.

Wesson oil was saponified by boiling 1500 gm. of the oil for about $\frac{1}{2}$ hour in 2000 cc. of 95 per cent alcohol in which 400 gm. of KOH were dissolved. The soap solution was acidified with sulfuric acid and the fatty acids were collected and washed with hot water until the washings no longer gave the test for the sulfate ion with barium chloride. Generally five to six washings were sufficient to accomplish this, although as many as ten washings were sometimes necessary.

The fatty acids were then distilled in a vacuum, which was obtained with a mercury-vapor pump, at a temperature of 185–205°. To the distilled fatty acids, redistilled glycerol was added, an excess of 50 per cent over that required by theory being used. These were placed in a flask in an oil bath, the temperature

of which was maintained at about 200°.   Dry carbon dioxide was passed through the fatty acid-glycerol mixture to remove the water formed in the process of esterification.   The esterification was carried out in this manner for 5 to 6 hours.   Generally, 80 to 85 per cent of the fatty acids were esterified during this procedure.

The mixture was then subjected to heating at a temperature of 215–220° for about 4 hours in a high vacuum, obtained with a mercury-vapor pump.

In this manner it was possible to obtain an oil which contained only 2.5 to 4 per cent of free fatty acids as determined by titration and calculated as oleic acid.   This oil no longer had the uniform consistency of the original cottonseed oil at room temperature, but a white solid layer always settled out.

In the above procedures there are at least four treatments, any one of which should have removed or destroyed antineuritic vitamin B.   The four are, (1) solubility in hot acidulated water, (2) destruction with alkali, (3) loss by distillation—the antineuritic vitamin B not being known to be volatile, (4) very high temperatures employed for a period of from 8 to 10 hours.

As Fig. 5 shows, when no yeast is added as a source of antineuritic vitamin B, a pronounced sparing action is observed in the presence of our so called "synthetic" Wesson oil, which judging by the severity of the treatment it received should be entirely free from the antineuritic vitamin B.   Essentially the same results are obtained as with the original Wesson oil.   Little or no effect is seen at the 10 per cent level of the oil while a marked response takes place at the 51 per cent level.   At this level the response is slightly inferior to that given by the untreated Wesson oil.

When a low level of the antineuritic vitamin B was fed in the form of 50 mg. of brewers' yeast, 10 per cent of the "synthetic" oil brought about a marked response, equal, if not superior, to that brought about by the untreated Wesson oil.   A still greater improvement was brought about by raising the "synthetic" oil to 51 per cent.   Little or no difference is observed between the two oils at the high level.   In the presence of higher levels of the antineuritic vitamin B (200 and 800 mg. of brewers' yeast) the response is the same with the "synthetic" Wesson oil as with the

untreated Wesson oil, the maximum response occurring at the level of 10 per cent of these oils.[4]

It must, therefore, be considered as highly improbable that the sparing action of fat on the antineuritic vitamin B is due to the actual presence of this vitamin as an impurity in the fat. It would appear that this is a phenomenon displayed by fat *per se*. The physiological function which we must thus assign to fats is in the present state of our knowledge inexplicable.

### Fat Does Not Appreciably Spare the Heat-Stable Water-Soluble Vitamin.

It became of interest to us to determine whether the sparing action of fat was also exerted on the heat-stable water-soluble factor in autoclaved yeast.

In this work (Fig. 6) adequate antineuritic vitamin B was supplied all groups by an 85 per cent alcoholic extract of rice bran.[5]  One group of animals was given none of the heat-stable water-soluble factor in autoclaved yeast, one group 50 mg. daily of autoclaved yeast, and a third group twice this amount. Lard was the only fat used, and it was fed at levels of 10 and 50 per cent. As Fig. 6 indicates, there is no longer the very marked superiority of the groups receiving fat, so evident in the work in which the level of the antineuritic vitamin B was varied.

It must, however, be admitted that the groups receiving the highest level of fat were somewhat improved, both in growth and in the absence of gross symptoms of disease.[6]  No effort was made

[4] As the figures show, at the higher levels of the two oils inferior growth was obtained. In both cases diarrhea accompanied this inferior growth but with greater severity in the animals receiving the "synthetic" Wesson oil.

[5] Fresh rice bran was supplied us gratis by Rosenberg Brothers and Company of San Francisco. 1 kilo of bran was shaken several times daily for a period of at least 48 hours with 4 liters of 85 per cent alcohol. It was filtered and concentrated in a vacuum. The total extract generally obtained was about $\frac{3}{4}$ of the original volume used. We, therefore, considered the extract as derived from or equivalent to $\frac{3}{4}$ of the rice bran used. The equivalent of 8 gm. of rice bran was fed daily to supply the antineuritic vitamin B (Extract 8 FB).

[6] The symptoms appearing in our animals on a diet low in the heat-stable water-soluble vitamin can be described as follows· The eyelids are at

to determine whether such improvement as was noted with 50 per cent of lard, was due to vitamin impurity in the lard.   The reader will note a truly remarkable variation in the behavior of individual animals in every group, a variation, which save for the phenomenon of "refection," is without parallel in any dietary experiments known to us.   A repetition of this work has yielded essentially the same results.   As a whole, therefore, our work indicates that, contrary to the situation with the antineuritic vitamin B, there is little sparing action of fat on heat-stable water-soluble vitamin.

<div align="center">DISCUSSION.</div>

So far as we know, no definite physiological function has been assigned to fats.   In general, investigators have looked upon fats as contributing only in the energy they supplied to the diet.   The data presented leave little doubt that fats as such exert an important function in the metabolism of the animal other than supplying energy.   Fats in common with substances carrying antineuritic vitamin B are able to delay or avert entirely the appearance of symptoms known as beriberi or polyneuritis.   This is most marked on diets in which sugar forms the source of energy, but it is also

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first swollen, then the hair around the lids is lost, presenting bare rings surrounding the eyes, the condition described as "spectacles."   This bareness spreads from a circumocular zone until at times almost the whole face is free from hair.   The skin is thin and red.   Very often sores are noted around the mouth.   We have not observed lesions under the tongue as have been described.   Diarrhea often accompanies the deficiency. Very often bloody vibrissæ are noted.   The bloody vibrissæ are present where no lesions are seen about the mouth to offer a possible source of the blood, though it must be remembered that slight lesions are often present on the paws, and blood on vibrissæ may be due to the animal's attempts to clean itself.   We have also had occasion to observe animals with the classical bilateral lesions on the back and sides, though this is not very common. Lesions on the nose are very common; these are sometimes accompanied by scaliness, though this is not always the case.   Since scaliness has been so frequently described as characteristic of the deficiency, due to absence of the heat-stable water-soluble factor present in autoclaved yeast, it might be mentioned that we have observed it in animals in which there was an abundance of autoclaved yeast and that the sign is thus hardly pathognomonic of this deficiency.   The symptoms did not supervene in all groups, nor in all animals in each group.

evident when starch is the source of energy. The activity of fats has been observed in this laboratory regardless of the level of antineuritic vitamin B extracts fed. To be sure, the sparing activity of fats is most marked when low levels of antineuritic vitamin B are fed. Whether fat no longer exerts a beneficent influence when antineuritic vitamin B has been supplied in sufficiently large amounts, must be left as an open question. The answer to this question is of great importance, for only then shall we know whether fat acts as a sparer of antineuritic vitamin B only or has other special and unknown uses in purified diets. All we can say here at present is that we have as yet found no case where fat did not improve the diet slightly after we had supposedly satisfied the antineuritic vitamin B requirements with unusually high levels of this vitamin. This question is at present under investigation.

Recent work on refection by Fridericia and others has brought to the fore the rôle that organisms may play in the digestive tract of animals and their importance in all vitamin B work. We have been led to wonder whether or not fats may be acting indirectly through their beneficial effect on the microorganisms in the intestinal tract of the rat. The question must be left open. We must, however, point out that the data here shown present such a quantitative picture of the activity of fat as to differ markedly from refection, for there is little uniformity or quantitative aspect to refection, nor has refection so far as we are aware been shown possible on diets such as we have employed. The data on the influence of fats on the course of the deficiency due to the absence of the heat-stable water-soluble vitamin strongly suggest that the two vitamins differ fundamentally in their relation to the presence of fat in the diet.

All natural fats studied by us have shown the sparing action on the antineuritic vitamin B and we have used butter, corn oil, coconut oil, and walnut oil besides those previously specifically mentioned. The influence of physical and chemical properties of the fats (melting point, degree of saturation, etc.) on their ability to act in the way herein described is being investigated.

*Components of Diets Used in Experiments Represented in Figs 1 to 3.*
*No Fat.*

Diet 542.

| | |
|---|---|
| Casein (L-3) | 20 |
| Autoclaved yeast | 10 |
| Sugar | 70 |
| Salts | 4 |

*10 Per Cent Fat.*

| Diet 550. | | Diet 563. | | Diet 564. | |
|---|---|---|---|---|---|
| Casein (L-3) | 20 | Casein (L-3) | 23 | Casein (L-3) | 23 |
| Autoclaved yeast | 10 | Autoclaved yeast | 10 | Autoclaved yeast | 10 |
| Sugar | 59 | Sugar | 56 | Sugar | 56 |
| Salts | 4 | Salts | 4 | Salts | 4 |
| Lard | 10 | Crisco | 10 | Wesson oil | 10 |

*50 to 51 Per Cent Fat.*

| Diet 559. | | Diet 571. | | Diet 572. | |
|---|---|---|---|---|---|
| Casein (L-3) | 36 | Casein (L-3) | 38 | Casein (L-3) | 36 |
| Autoclaved yeast | 10 | Autoclaved yeast | 10 | Autoclaved yeast | 10 |
| Salts | 4 | Salts | 4 | Salts | 4 |
| Lard | 50 | Crisco | 55 | Wesson oil | 55 |

All diets were supplemented with 2 drops of cod liver oil daily.

*Components of Diets Used in Experiments Represented in Fig. 4.*

| Diet 542. | | Diet 414. | | Diet 415. | |
|---|---|---|---|---|---|
| Casein (L-3) | 20 | Casein (L-3) | 19 | Casein (L-3) | 20 |
| Autoclaved yeast | 10 | Autoclaved yeast | 10 | Autoclaved yeast | 10 |
| Sugar | 70 | Corn-starch | 67 | Corn-starch | 59 |
| Salts | 4 | Salts | 4 | Salts | 4 |
| | | | | Lard | 10 |

All diets were supplemented with 2 drops of cod liver oil daily.

*Components of Diets Used in Experiments Represented in Figs. 5 and 6.*
*No Fat.*

| Diet 540. | | Diet 542. | |
|---|---|---|---|
| Casein (L-3) | 25 | Casein (L-3) | 20 |
| Sugar | 75 | Autoclaved yeast | 10 |
| Salts | 4 | Sugar | 70 |
| | | Salts | 4 |

*10 Per Cent Fat.*

| Diet 551. | | Diet 564. | | Diet 565. | |
|---|---|---|---|---|---|
| Casein (L-3)..... | 27 | Casein (L-3) ... | 20 | Casein (L-3).... | 27 |
| Sugar.... ....... | 59 | Autoclaved | | Autoclaved | |
| Salts............. | 4 | yeast ........ | 10 | yeast........ | 10 |
| Lard............. | 10 | Sugar........... | 56 | Sugar........... | 56 |
| | | Salts............ | 4 | Salts .......... | 4 |
| | | Wesson oil...... | 10 | "Synthetic" | |
| | | | | Wesson oil.... | 10 |

*51 to 54 Per Cent Fat.*

| Diet 572. | | Diet 573. | | Diet 579. | |
|---|---|---|---|---|---|
| Casein (L-3)..... | 38 | Casein (L-3).... | 38 | Casein (L-3).... | 40 |
| Autoclaved | | Autoclaved | | Salts............ | 4 |
| yeast ......... | 10 | yeast ........ | 10 | Lard........... | 53 |
| Salts............ | 4 | Salts... ........ | 4 | | |
| Wesson oil ...... | 55 | "Synthetic" | | | |
| | | Wesson oil.... | 55 | | |

All diets were supplemented with 2 drops of cod liver oil daily. Diets 540, 551, and 579 were supplemented with 1 cc. daily of an alcoholic extract of rice bran (Extract 8 FB).
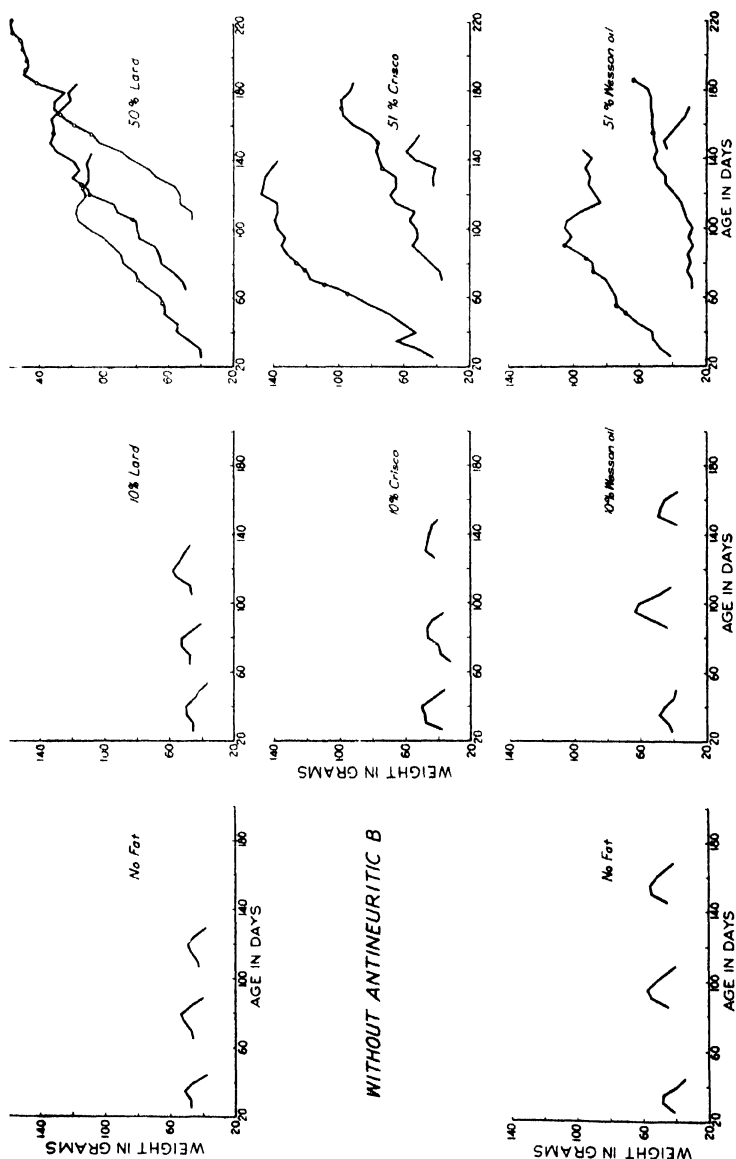
FIG. 1. Growth of animals when no antineuritic vitamin B was added to diets containing no fat (Diet 542), 10 per cent fat (Diets 550, 563, 564), or higher levels of fat (Diets 559, 571, 572).
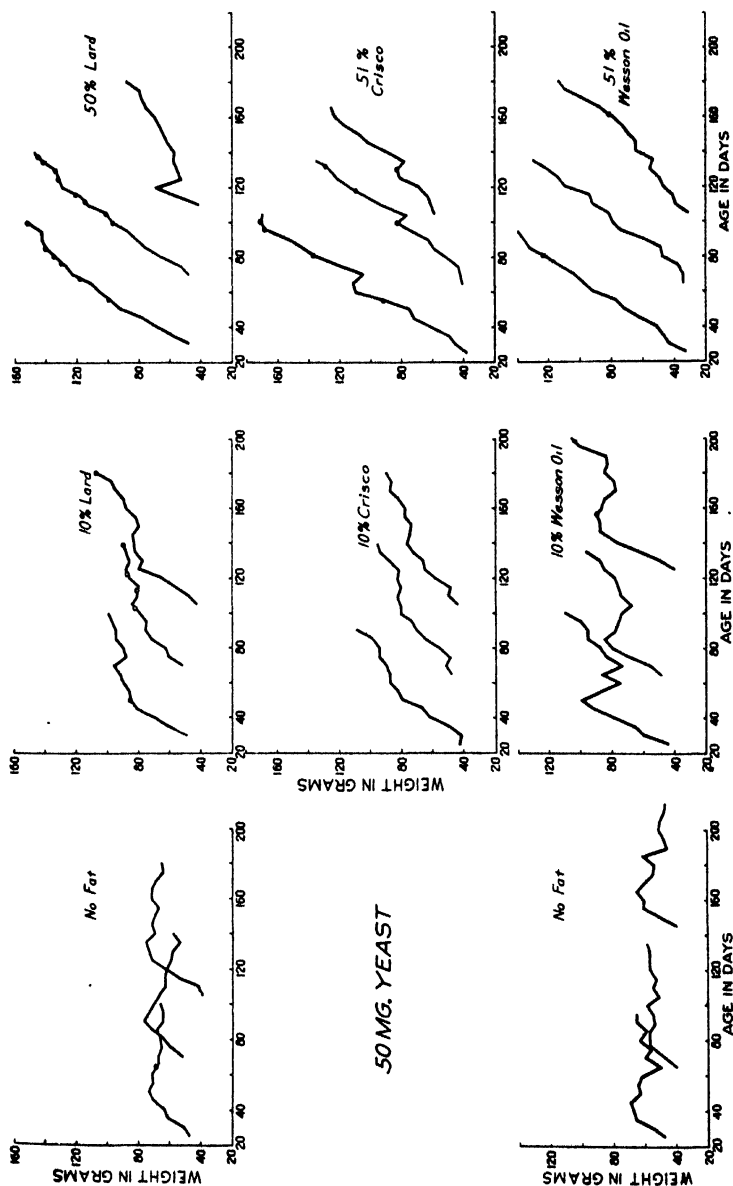
FIG. 2. Growth of animals when 50 mg. of brewers' yeast were added as the source of antineuritic vitamin B to diets containing no fat (Diet 542), 10 per cent fat (Diets 550, 563, 564), or higher levels of fat (Diets 559, 571, 572).
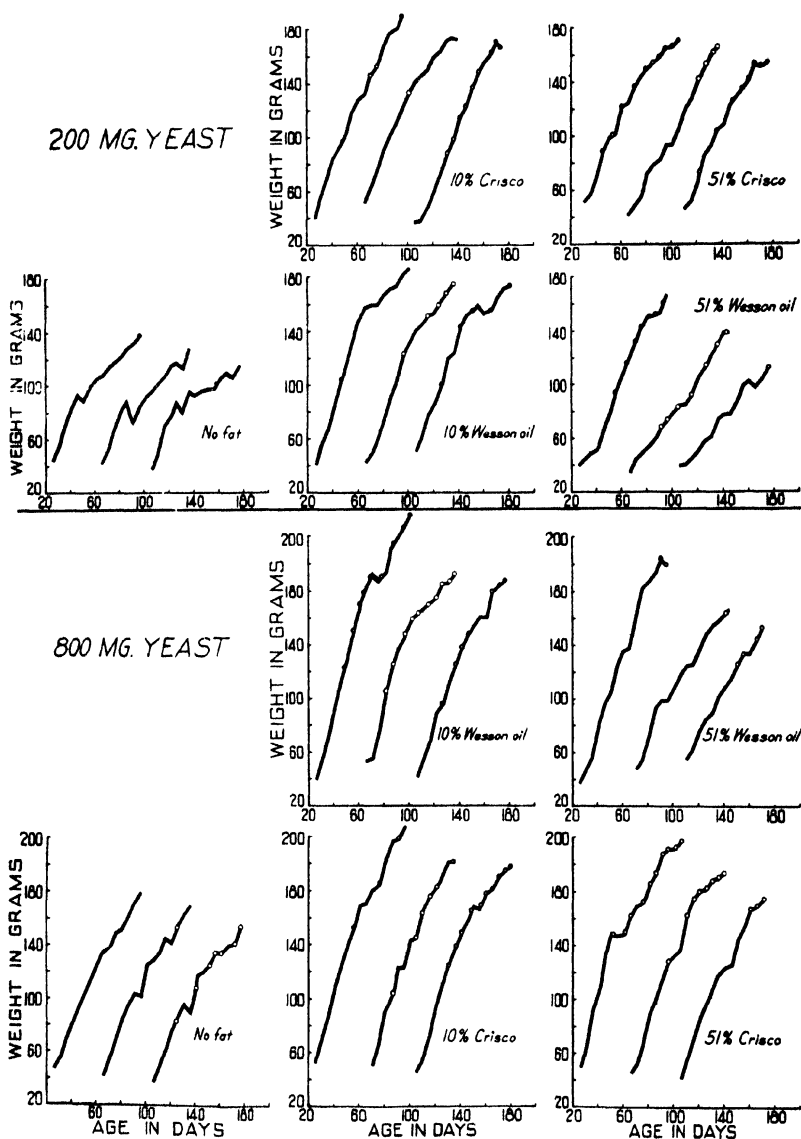
FIG 3. Growth of animals when higher levels of antineuritic vitamin B (200 mg. and 800 mg. of brewers' yeast) were added to diets containing no fat (Diet 542), 10 per cent fat (Diets 563, 564) and 51 per cent fat (Diets 571, 572)
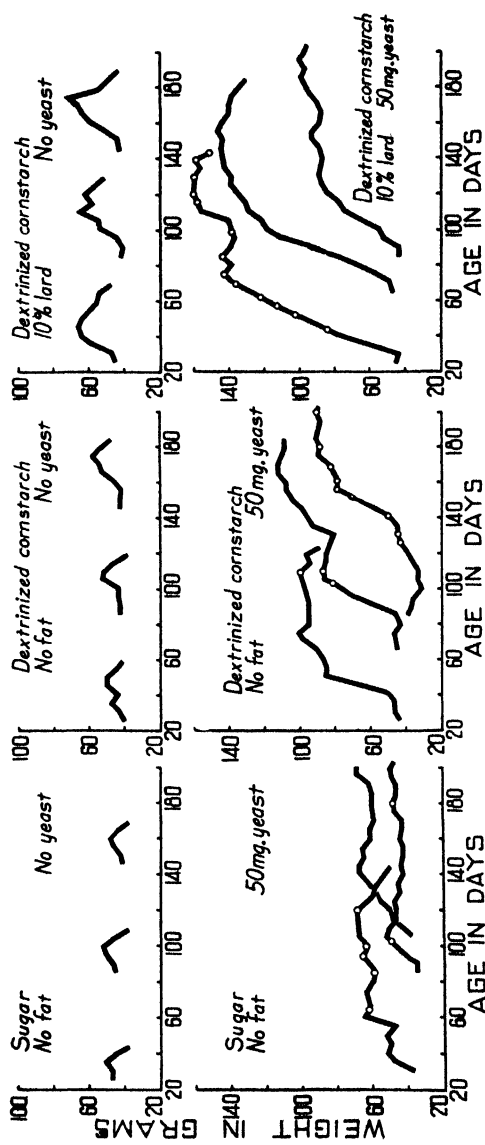
Fig. 4. Growth of animals on diets in which the sugar (Diet 542) had been replaced by dextrinized corn-starch (Diet 414). The diet with corn-starch gave superior growth. The addition of fat to this diet (Diet 415) further improved the growth, illustrating the sparing action of fat in the presence of this carbohydrate. These differences were most marked when 50 mg. of brewers' yeast were added as the source of antineuritic vitamin B.
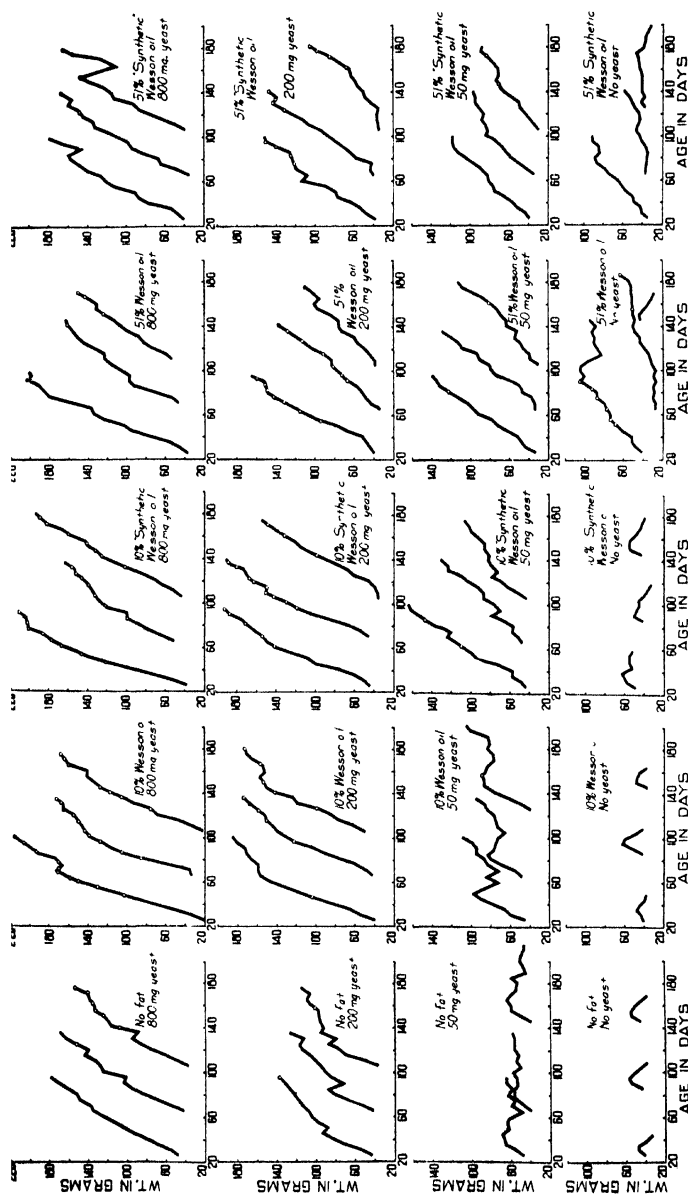
FIG. 5. Growth of animals on a natural fat (Wesson oil) and the same fat subjected to saponification, distillation, and reesterification ("synthetic" Wesson oil). Four levels of antineuritic vitamin B were added to the diets, one group receiving no yeast, a second group 50 mg., a third group 200 mg., and the fourth 800 mg. of brewers' yeast. Diets: no fat (Diet 542), 10 per cent Wesson oil (Diet 564), 10 per cent "synthetic" Wesson oil (Diet 565), 51 per cent Wesson oil (Diet 572), 51 per cent "synthetic" Wesson oil (Diet 573).
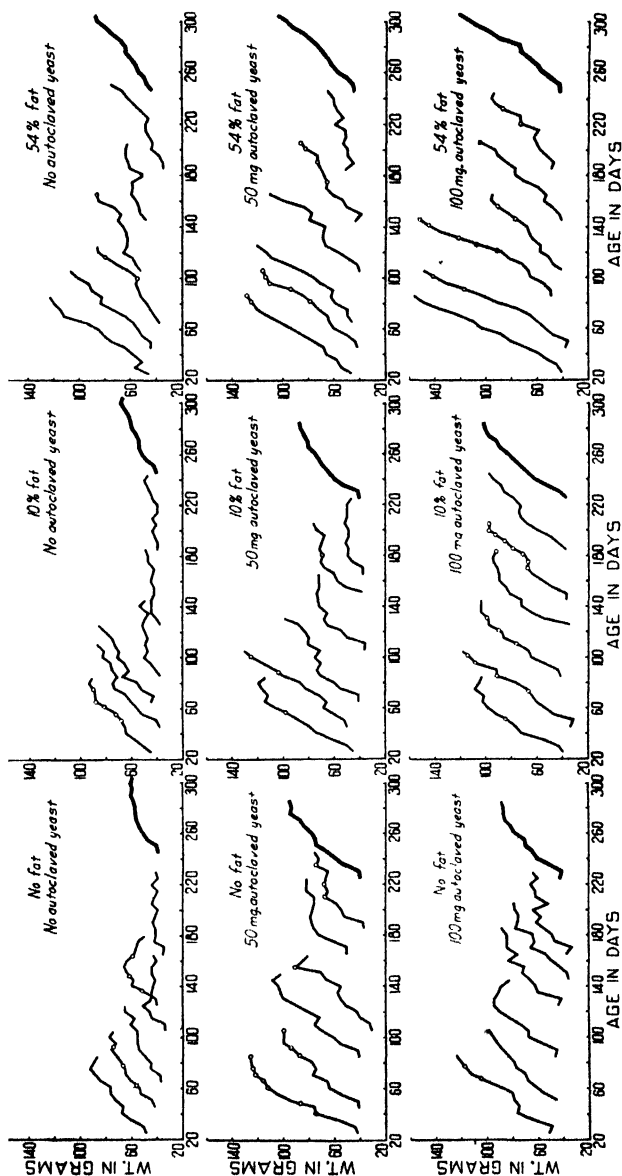
FIG. 6. Growth of animals upon diets containing inadequate amounts of the thermostable vitamin in the form of autoclaved yeast—one group receiving no yeast, a second 50 mg., a third 100 mg. of autoclaved yeast. The animals of all groups received daily an adequate amount of the antineuritic vitamin B in the form of 1 cc. of an alcoholic extract of rice polishings (Extract 8 FB). The diets had no fat (Diet 540), 10 per cent fat (Diet 551) and 54 per cent fat (Diet 579). The heavy curve in each instance is a composite for the group of six.

# A PREVIOUSLY UNDETECTED CONSTITUENT OF BLOOD.

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In protein-free tungstic acid blood filtrates, besides uric acid, are other substances which give a blue color with alkaline arseno-phosphotungstate. Bulmer, Eagles, and Hunter (1) demonstrated the presence of such a substance. It was subsequently· isolated by Hunter and Eagles (2, 3) and was shown to be ergothioneine by Benedict and coworkers (4, 5, 6) and Eagles and Johnson (7). The name thioneine was suggested by Benedict (6) for the substance.

Acid hydrolysis of the protein-free filtrate liberates uric acid (Benedict and coworkers (8–11)). Evidence is here presented that acid treatment of the thioneine fraction increases the material measured by the arsenophosphotungstate reaction. Provisionally the substance so produced has been named substance Z.

Since uric acid, thioneine, and substance Z give the same blue color it seemed desirable to determine their quantities. Oxalated blood was used in all cases and four colorimetric determinations were made on each sample; (1) uric acid direct,[1] (2) uric acid indirect, (3) thioneine plus substance Z,·(4) thioneine. A part of the blood filtrate was hydrolyzed with sulfuric acid, a part was not so treated; uric acid, both direct and indirect, was determined in the unhydrolyzed portion. Substance Z is freed by acid hydrolysis and is then precipitated by silver lactate but is not afterwards liberated by acid NaCl as used by Folin and Wu (12). Hence it is determined with thioneine in the silver residue from the hydrolyzed portion. Thioneine is similarly determined in the unhydrolyzed portion.

Other than the usual reagents for uric acid determination there

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[1] The terms "direct" and "indirect" are used with the significance given them by Bulmer, Eagles, and Hunter (1).

are: (1) concentrated sulfuric acid diluted 1:10 by volume; (2) ammoniacal sodium acetate solution, containing 300 cc. of concentrated $NH_4OH$ with 700 cc. of 4 N $NaC_2H_3O_2$; (3) silver lactate solution. Mix 50 cc. of 10 per cent NaOH and 50 cc. of 85 per cent lactic acid and add to 50 gm. of silver lactate (Merck) previously dissolved in 350 cc. of warm water. Bring to 1000 cc. and keep in the dark for at least 6 weeks before using. It must be clear and colorless.

*Procedure.*

To 20 cc. of Folin and Wu blood filtrate in a 50 cc. centrifuge tube add 7 cc. of silver lactate solution. Let it stand 2 or 3 minutes, centrifuge, and decant the supernatant liquid. To the residue add 1 cc. of 10 per cent NaCl in 0.1 N HCl, stir with a small glass rod, add 4 cc. of water, and centrifuge again. Decant the supernatant liquid into a clean, dry test-tube. Repeat the treatment with acid NaCl and water and the centrifugation, adding the liquid to the portion previously obtained. Dilute this mixture with distilled water to 20 cc., the volume of the original blood filtrate. Transfer 5 cc. of the mixed liquid to another clean, dry test-tube, add 5 cc. of water, 4 cc. of 5 per cent NaCN, 1 cc. of Benedict's uric acid reagent, and develop the color in the usual manner. This represents indirect uric acid. Direct uric acid is determined in the Folin and Wu blood filtrate according to Benedict (13).

For the thioneine determination add 4 cc. of 5 per cent NaCN to the silver residue from the second treatment with acid NaCl and stir until completely dissolved. Decant into a clean, dry test-tube; rinse twice with 5 cc. portions of water, adding each portion to the decanted cyanide, then add 1 cc. of uric acid reagent and carry out the colorimetric determination as for uric acid. A 0.02 mg. standard is generally used though sometimes a 0.01 mg. one (Benedict (13)) is preferable. The standard tube is set at 15 and the thioneine, in terms of uric acid, is calculated by

$$\frac{15}{R} \times 0.02 \times \frac{100}{2} = \frac{15}{R} = \text{mg. of thioneine in 100 cc. of blood when}$$

$R$ is the reading of the unknown and 0.02 mg. the uric acid standard used.

For the determination of thioneine plus substance Z 15 cc. of

Folin and Wu blood filtrate are transferred to a test-tube (1 × 8 inches) for hydrolysis and 3 cc. of the diluted sulfuric acid are added.    As an air condenser a glass tube (200 cm. × 8 mm.) is attached to the test-tube by a 1-hole rubber stopper.    The contents of the tube are gently boiled for 15 minutes over a low flame (a micro burner may be used).    After cooling, giving time for complete drainage of the condenser before disconnecting, shake so as to wash down any liquid which may have condensed in the upper part of the test-tube.    The volume of the liquid must be the same as before hydrolysis, 18 cc.

To the hydrolyzed blood filtrate add from a 10 cc. burette 2 to 3 cc. of the ammoniacal sodium acetate solution.    The amount required is previously found by titrating 15 cc. of protein-free filtrate mixed with 3 cc. of the sulfuric acid until faintly alkaline to Congo red.    2.5 cc. of the acetate solution will usually be found correct; it is best to check it occasionally especially after preparing new reagents for precipitation of the blood proteins.    Mix and pour into a centrifuge tube, rinsing with 5 cc. of water.    Treat this solution as described in the method for the determination of indirect uric acid and thioneine except that the acid NaCl washings are discarded and thioneine plus substance Z determined from the silver residue remaining.    Amounts are calculated from $\frac{15}{R} \times 0.02 \times \frac{100}{1.5}$ = mg. of thioneine + substance Z in 100 cc. of blood where $R$ is the reading of the unknown and the 0.02 mg. standard is set at 15 mm.    Subtracting the amount of thioneine found in the unhydrolyzed filtrate from the above gives the amount of substance Z.

The development of color in all four determinations is made at the same time and compared against the same standard.    If read immediately after cooling no turbidity results.    The maximum limit of error permitted in duplicate determinations was 0.1 mg. in 100 cc.

The blood was drawn from the cubital vein, oxalated, and treated immediately with tungstic acid.    Ample time was allowed for complete precipitation.    The results for fifteen normal bloods are given in Table I.    They are expressed (as are all others cited) in terms of mg. of uric acid in 100 cc. of blood.

Of the thirty-eight normal human bloods examined only one

TABLE I.

*Uric Acid, Thioneine, and Substance Z in Normal Human Blood.* *

| Subject No. | Uric acid. | | Thioneine. | Substance Z. |
|---|---|---|---|---|
| | Direct method. | Indirect method. | | |
| 1 | 4.4 | 3 2 | 1.1 | 0.5 |
| 2 | 3.0 | 2.4 | 0.6 | 0.7 |
| 3 | 3.6 | 2.6 | 1.1 | 0.4 |
| 4 | 4.3 | 3.3 | 0.8 | 0.5 |
| 5 | 3.5 | 2.9 | 0.7 | 1.1 |
| 6 | 3.6 | 3.5 | 0.0 | 0.9 |
| 7 | 4.0 | 4.0 | 0.0 | 0.5 |
| 8 | 3.3 | 2.8 | 0.7 | 0.3 |
| 9 | 3.5 | 3.3 | 0.7 | 0.2 |
| 10 | 3.4 | 3.2 | 0.8 | 0.3 |
| 11 | 4 2 | 3.8 | 1.1 | 0.6 |
| 12 | 3.2 | 3.2 | 0.5 | 0.4 |
| 13 | 2 7 | 2 3 | 0.9 | 0.4 |
| 14 | 2 9 | 2 6 | 0.7 | 0.0 |
| 15 | 3.6 | 3 5 | 0.8 | 0.4 |
| Average . . . | 3.5 | 3 1 | 0.7 | 0.5 |
| "   of   38 samples . . . . . | 3.3 | 2.8 | 0.7 | 0.5 |

* Results are expressed as mg. of uric acid per 100 cc. of blood.

TABLE II.

*Thioneine and Substance Z in Pathological Human Blood.*

| Diagnosis. | No. of cases. | Thioneine. | | Substance Z. | |
|---|---|---|---|---|---|
| | | Range. | Average. | Range. | Average. |
| Pernicious anemia . . . . . . . . . . . . | 8 | 0.4–1.0 | 0.6 | 0.2–1.8 | 1.2 |
| Leucemia. . . . . . . . . . . . . . . . . . . . . | 3 | 0.7–0.8 | 0.7 | 0.7–1.8˙ | 1.1 |
| Nephritis. . . . . . . . . . . . . . . . . . . . . | 4 | 0.5–1.0 | 0.7 | 0.0–1.0 | 0.4 |
| Diabetes mellitus. . . . . . . . . . . . . . | 4 | 0.8–1.2 | 1.0 | 0.6–1.3 | 0.9 |
| Arsenical dermatitis. . . . . . . . . . . . | 3 | 0.8–1.2 | 1.0 | 0.1–1.6 | 0.8 |
| Primary   lues. . . . . . . . . . . . . . . . . | 9 | 0.5–1.0 | 0.7 | 0.1–1.4 | 0.6 |
| Secondary • " . . . . . . . . . . . . . . . . . | 9 | 0.6–1.0 | 0.7 | 0.1–1.0 | 0.4 |
| Tertiary   "   . . . . . . . . . . . . . . . . . | 4 | 0.5–0.9 | 0.6 | 0.1–1.5 | 0.8 |
| Latent   "   . . . . . . . . . . . . . . . . . | 4 | 0.7–1.0 | 0.8 | 0.2–1.5 | 0.7 |
| Paresis. . . . . . . . . . . . . . . . . . . . . . | 2 | 0.5–0.4 | 0.4 | 0.8–0.8 | 0.8 |

failed to give a test for substance Z. Twenty-nine contained more than 0.2 mg. (in terms of uric acid) in 100 cc. Eighteen showed 0.5 mg. or more; the highest was 1.1 mg. It will be seen that the level of substance Z is not related to the thioneine or uric acid content, nor is it related to age or sex, though in most

TABLE III.

*Uric Acid,\* Thioneine, and Substance Z in Animal Blood and Tissues.*

| Species. | Tissue. | Uric acid. | Thioneine. | Substance Z. | Species. | Tissue. | Uric acid. | Thioneine. | Substance Z. |
|---|---|---|---|---|---|---|---|---|---|
| Dog   I | Blood. | 1.0 | 0.4 | 0.6 | Rabbit XI | Muscle. | 1.4 | 0.5 | 0.2 |
| "   II | " | 1.2 | 0.5 | 0 2 | | Kidney. | 2 9 | 1.1 | 0.2 |
| "  III | " | 1.1 | 0.4 | 0.5 | | Liver. | 8.0 | 1.0 | 0.8 |
| "  IV | " | 1.1 | 0.4 | 0.1 | | | | | |
| "   V | " | 1.1 | 0.5 | 0.6 | "   XII | Blood. | 1.0 | 0.5 | 0.1 |
| | | | | | | Muscle. | 1.3 | 0.4 | 0.3 |
| "  VI | Muscle. | 2.7 | 1.3 | 0.5 | | Kidney. | 2.8 | 1.5 | 0.3 |
| | Liver. | 6.0 | 2.1 | 0.5 | | Liver. | 8.2 | 1.5 | 0 4 |
| | Kidney. | 6.7 | 4.7 | 0.5 | | | | | |
| | | | | | "  XIII | Blood. | 1.2 | 0 8 | 0.2 |
| " VII | Muscle. | 3.7 | 2.4 | 0.2 | | Muscle. | 1 6 | 0.5 | 0 2 |
| | Liver. | 6 4 | 3 5 | 1.2 | | Kidney. | 2.9 | 1.2 | 0.1 |
| | Kidney. | 8.0 | 6 8 | 0 2 | | Liver. | 5.4 | 1.3 | 0.5 |
| | | | | | | | | | |
| Rabbit  I | Blood. | 1.3 | 0 6 | 0.2 | Pig  I | Blood. | 1.0 | 0.6 | 0.1 |
| "   II | " | 1 1 | 0.4 | 0.1 | "  II | " | 1.5 | 0.9 | 0.2 |
| "  III | " | 1.7 | 1 0 | 0 5 | | Liver. | | 1.9 | 0.6 |
| "  IV | " | 1.4 | 0 6 | 0 1 | | | | | |
| "   V | " | 1 5 | 0 5 | 0 8 | Ox  I | Blood. | 2 0 | 0 5 | 0.2 |
| | | | | | "  II | " | 2.1 | 0.5 | 0 1 |
| | | | | | " III | " | 1 8 | 0.4 | 0 2 |
| | | | | | " IV | " | 2 3 | 0 4 | 0 3 |

\* As determined by Benedict's direct method.

cases the content of female blood is low. The distribution of substance Z between plasma and corpuscles appears to be equal.

The results of analyses of pathological bloods are shown in Table II.

The range of thioneine and substance Z content is recorded because of the belief that the average is of less significance. No path-

ological case was examined which gave consistently a high or low value for substance Z although in both pernicious anemia and leucemia the general trend was toward a high level.    There was no correlation of these findings with either the white or the red blood cell counts.    It is of interest to know that in nephritis the level of substance Z tends to be low, while not paralleling the non-protein nitrogen.    In several cases of infectious lues the amount of substance Z in the blood decreased markedly with the administration of organic arsenicals, where it was high before treatment.    Often where the amount of substance Z was 0.6 mg. per 100 cc. or less no such change occurred.    In diabetes the substance Z content of the blood did not parallel the blood sugar level.    In this connection it may be noted that addition of urea and dextrose to the blood in five or six times the normal amount does not influence the substance Z content.    Rabbits infected with *Treponema pallidum* showed a substance Z content of the blood no different from the normal determined before infection.    The arsenicals. arsphenamine and stovarsol, when administered to rabbits failed to produce any change in substance Z content.

For comparison with human blood similar determinations were made on animal bloods; their tissues were also tested.    Dog and rabbit blood was obtained by cardiac puncture; pig and ox blood came fresh from the abattoir as did .also pig liver.    All tissue filtrates were prepared from the still warm organs by the method of Folin, Berglund, and Derick (14).    The results are recorded in Table III.

It is apparent that the amount of substance Z in either blood or tissue filtrates is in no way related to that of thioneine or uric acid (by the direct method).    There is no distinct difference in its level in the blood of man, dog, or rabbit.    In the few specimens of pig and ox blood examined the amount found was small. Muscle and kidney contain about equal amounts, liver considerably more.    Severe liver injury produced with hydrazine sulfate in rabbits failed to alter the level of substance Z in any of the tissues.

The substance Z content of human blood is not altered by sterile or non-sterile incubation for 24 hours at 40°.    As seen from the observations recorded in Table IV it increases during incubation of rabbit or pig liver without a preservative though no such

increase appeared in rabbit blood, muscle, or kidney. In incubation with a preservative a 5 gm. portion was ground with quartz sand, then suspended in water to 90 cc. under toluene.

## DISCUSSION.

Substances which will reduce phosphotungstic acid reagent are not limited to uric acid and thioneine. Among such active sub-

TABLE IV.·

*Thioneine and Substance Z Fractions in Preserved and Incubated Liver Tissue.*

| Materials and conditions. | Thioneine fraction. | Substance Z. |
|---|---|---|
| Pig liver, fresh from abattoir.        ..... . ...... .. | 2 0 | 0.5 |
| "    "    kept at  10° for 72 hrs        ... . .... . | 2 0 | 0 5 |
| "    "    "    "  −5°  " 72  "  ............... ... | 2 4 | 0 5 |
| "    "    incubated under toluene for 24 hrs. at 37°. | 3 6 | 0.6 |
| "    "    "    "    "    " 72  "  " 37°.. | 4 0 | 0.7 |
| Pig liver, fresh from abattoir   ... . . .... . | 1 9 | 0 6 |
| Pig liver, incubated 24 hrs. at 37° without preservative  .  ..... ................................... | 3.1 | 1.7 |
| Pig liver, incubated 72 hrs. at 37° without preservative... .  ....................................... | 2.7 | 1.4 |
| Pig liver, kept at −5° for 72 hrs. then incubated 24 hrs. at 37° without preservative... ..... ............. | 2.7 | 1.4 |
| Rabbit liver, fresh from animal .  ..  .. ............. | 1.5 | 0.4 |
| "    "    kept 72 hrs. at 10°. . .................. | 2.6 | 0.7 |
| "    "    incubated 12 hrs. at 37°, no preservative. | 2.2 | 0.9 |
| "    "    kept at 10° for 72 hrs. then incubated 12 hrs. at 37°..... .  ................................ | 3 6 | 1 0 |

stances have been pointed out metallic silver (13), cystine after reduction to cysteine (15), and glutathione (16) as well as insulin (17).

It has been shown (12) that acid NaCl quantitatively removes uric acid from the silver precipitate; inasmuch as substance Z is not thus removed it cannot be uric acid freed by acid hydrolysis from some combined form. Since glutathione is present in the blood as such it is evident that substance Z is not glutathione.

The possibility must be considered that it is cystine or cysteine, liberated by acid hydrolysis from glutathione, a dipeptide of cysteine and glutaminic acid. Hunter and Eagles (18) found for such hydrolysis of glutathione that it was necessary to boil for 8 hours with 6 N sulfuric acid; for the development of substance Z the heating was for 15 minutes with 0.6 N acid. Hunter and Eagles (19) found normal human blood corpuscles to contain in 100 cc. about 100 mg. of glutathione; Thompson and Voegtlin (20) showed that it is absent from the serum. Whole blood therefore contains approximately 50 mg. of glutathione per 100 cc. This 50 mg. represents potentially 24.1 mg. of cystine. The results obtained with the Folin and Wu filtrate to which cystine

TABLE V

*Effect of Added Cystine and Cysteine on Thioneine Fraction of Blood.*
Figures are in mg per 100 cc. of blood.

| Blood No | Cysteine added. | Cystine added. | Thioneine. |
|---|---|---|---|
| 1 a | 0 0 | | 0 6 |
| 1 b | 25 0 | | 0 8 |
| 1 c | | 25 0 | 0 8 |
| | | | |
| 2 a | 0 0 | | 0 7 |
| 2 b | 25 0 | | 0 9 |
| 2 c | 37 5 | | 1 0 |
| 2 d | 50 0 | | 1 1 |

or cysteine had been added show an increase of only 0.2 mg. (expressed in terms of uric acid) for 25 mg. added. Even if complete hydrolysis in 15 minutes were possible twenty-nine of the thirty-eight bloods examined contained more than 0.2 mg. of substance Z. Our distributional analysis showed no more substance Z in protein-free corpuscle filtrate than in plasma filtrate while glutathione does not occur in the latter (20). Sullivan's naphthoquinone test (21) was applied to the bloods of Table V. In the Folin and Wu filtrate, before or after hydrolysis it was negative; where cysteine was added it was positive. Consequently substance Z is neither glutathione nor cysteine split from it.

Du Vigneaud (17) obtained a blue color with hydrolysates of insulin materials but Shonle and Waldo (22) found that tungstic

acid filtrates contained no insulin, which eliminates that from consideration.

### SUMMARY.

A previously undetected substance, reducing arsenophospho-tungstic acid, is shown to be present after acid hydrolysis of the blood of man, dog, and rabbit.  It is also found in the muscle, kidney, and liver of dog and rabbit.  In the few ox and pig bloods examined it was detected only in traces.

It is provisionally named substance Z.

A method is described for its estimation in tungstic acid blood and tissue filtrates.  Its amounts have been determined in normal and various pathological bloods.

It is not the same as any of the known constituents of tungstic acid blood and tissue filtrates.

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# A NOTE ON THE CREATINE-CREATININE EXCRETION DURING FASTING.

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The problem of the nitrogen partition in the urine and of the creatine-creatinine elimination during fasting has been studied extensively and little of fundamental importance can be added to the already accumulated data. The literature of the subject has also been so well summed up in previous publications that it will not be repeated here. In presenting the results of studies on the nitrogen partition of fasting dogs carried out for several years in this laboratory I am prompted by a desire to put on record experimental material which in the main will serve to correct an erroneous conception concerning the creatine-creatinine excretion during inanition. In Tables I to III are presented the data which were obtained on a number of dogs fasted with or without water until they had lost from 31 to 40 per cent of their body weight. For several of these animals data are also available showing the effect of refeeding for periods lasting 6 to 9 days.

Before fasting each dog was fed for a time a uniform diet. This was essentially as recommended by Cowgill except that we have not been successful in feeding either casein or dry meat powder as the source of protein. Our animals generally refused to eat the mixture so prepared, or at best would accept it only for a few days. We finally had to use meat instead. We used beef heart thoroughly cleared from fat and connective tissue, finely ground, and washed in running water for about 24 hours. The washed meat was pressed dry and frozen solid in brine. This meat was analyzed and used in all the experiments except the first three.

The analytical procedure was based upon the methods as described in Folin's "Laboratory Manual." The ammonia was

TABLE I.

| Dog No. | Period. | | Change in body weight. | N partition in urine. | | | | Creatinine coefficient. |
|---|---|---|---|---|---|---|---|---|
| | | | | Total N. | NH₃-N | Creatinine N. | Creatine N. | |
| | | | *per cent* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 2,  13 05 kilos. | 1. P,* 3 days. | | | 28.00 | 1145 | 322 | 197 | 8.33 |
| | 1. F,* | 4 days. | −6.8 | 13.52 | 835 | 466 | 185 | 9.2 |
| | 2. " | 4 " | −11 8 | 11.11 | 938 | 432 | 295 | 9.2 |
| | 3. " | 4 " | −15.7 | 8.46 | 633 | 408 | 57 | 9.1 |
| | 4. " | 4 " | −19.4 | 10 08 | 731 | 442 | 93 | 10.3 |
| | 5. " | 4 " | −22.2 | 7.79 | 398 | 365 | 57 | 8.8 |
| | 6. " | 4 " | −25 6 | 7.50 | 438 | 335 | 72 | 8.5 |
| | 7. " | 4 " | −28 2 | 7.23 | 352 | 320 | 72 | 8.4 |
| | 8. " | 4 " | −30.7 | 6.22 | 420 | 168 | 268 | 4.6 |
| | 9. " | 4 " | −33 6 | 6.50 | 415 | 153 | 280 | 4.3 |
| 3,  12 50 kilos. | 1. P, | 3 days. | | 17.80 | 719 | 389 | 397 | 10 2 |
| | 1. F, | 3 days. | −5.5 | 7.42 | 416 | 335 | 237 | 8.8 |
| | 2. " | 3 " | −9 3 | 6.89 | 338 | 341 | 199 | 9.5 |
| | 3. " | 3 " | −11 6 | 7.63 | 376 | 298 | 198 | 8.6 |
| | 4. " | 3 " | −14 4 | 6.77 | 354 | 305 | 175 | 9.1 |
| | 5. " | 3 " | −17.1 | 5.74 | 302 | 298 | 84 | 9.2 |
| | 6. " | 3 " | −19.4 | 6.33 | 383 | 322 | 118 | 10.2 |
| | 7. " | 3 " | −22.2 | 6.32 | 341 | 306 | 34 | 10.0 |
| | 8. " | 3 " | −23.9 | 6 40 | 248 | 248 | 64 | 8.4 |
| | 9. " | 3 " | −25.9 | 5.70 | 413 | 231 | 100 | 8.0 |
| | 10. " | 3 " | −28.0 | 5 02 | 377 | 249 | 90 | 8.8 |
| | 11. " | 3 " | −30.8 | 3.92 | 173 | 202 | 65 | 7.4 |
| | 12. " | 3 " | −32.7 | 3.06 | 238 | 210 | 47 | 8.0 |
| | 1. R,* | 3 days. | +5.2 | 6.68 | 298 | 251 | 45 | 9.5 |
| | 2. " | 3 " | +13.2 | 16.10 | 903 | 268 | 244 | 9.5 |
| | 3. " | 3 " | +25.0 | 21.88 | 839 | 353 | 818 | 11.4 |

Food consumed during the preliminary period by Dog 2, 246 gm. of meat powder (35 gm. of N), 100 gm. of sucrose, 168 gm. of fat mixture,† and 1500 cc. of water.

Food consumed by Dog 3:

| | Preliminary period. | Refeeding period. | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| Ground heart, *gm* | 750 | 400 | 800 | 1200 |
| Dry bread meal, *gm* | 150 | 80 | 160 | 240 |
| Fat mixture,† *gm* | ? | 80 | 160 | 240 |
| Water, *cc* | 1725 | 830 | 1150 | 1565 |

* The letters P, F, and R, denote preliminary, fasting, and refeeding periods respectively. Dogs 7 and 9 fasted without water (also Dog 5, not included in the tables) but all the other dogs received water during their fast.

† The fat mixture was Cowgill's as mentioned in the text.

determined either by the permutit or by the aeration method. The total nitrogen was determined by the Kjeldahl macro method.

In their extensive studies on nitrogen partition in the urine of fasting dogs, Howe, Mattill, and Hawk (4) develop the idea that in advanced stages of inanition and just preceding the fatal termination of the fast the creatine excretion of the animal exceeds that of the creatinine. If the data are plotted for the excretion of creatinine and of creatine, the curves cross; the "creatine-creatinine crossing," according to these authors, being pathognomonic of the ultimate changes in the nitrogenous metabolism and a warning of the approaching death. This idea has been accepted by Palladin and Epelbaum (8) who in a recent investigation on fasting cats state that the curves of creatine and creatinine excretion present in the last stage of fasting a crossing over. In our eight[1] experiments with dogs we observed this crossing over in two instances only. It can be argued, of course, that our animals have not been fasted long enough to bring them to the point of death, which accounts for our failure to find this phenomenon of the "crossing over." On the face of it this argument would seem valid, but we propose to show that the crossing over is not a sign that the existence of the organism has already been jeopardized by fasting. Of the two dogs which show this crossing over of the creatine and creatinine curves, Dog 2 (Table I) shows this at the time of a 30 per cent loss in body weight, and the crossing stands in no relation to a premortal rise in total nitrogen excretion which is supposed to occur at the same time. This dog was still in excellent condition when the inanition experiment was discontinued. Dog 9 (Table II), which likewise shows this crossing of the creatine and creatinine curves, had lost at that time only 33 per cent in weight. The animal died owing to an accident when a sample of blood was taken from its heart. This dog, however, was actually sick with distemper for the last few days and the urine contained considerable quantities of albumin. Nevertheless, even in this instance the crossing over can hardly be regarded as a warning that the animal was in extremis.

The results of our experiments with fasting dogs suggest, therefore, that the crossing over of the curves of creatine and

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[1] Detailed data for only five of these animals are given in the tables.

creatinine excretion may be purely accidental and bears no actual relationship to the premortal condition of the animal.   With this in view we examined the recent data of Palladin and Epelbaum (8) who so definitely accepted the hypothesis of the incidence of a crossing over in the terminal inanition stages.   Plotting the results of Palladin and Epelbaum's experiments on six cats made it possible to place more or less accurately the time of the crossing over of the two curves.   The following is the outcome of our study of their data.   In Cat 1 a crossing over occurs on the 17th day of the fast, when a loss of about 34 per cent in weight has been incurred, and from that time on the creatine curve rises continually and that of creatinine tends downward.   This cat was killed on the 24th day, when it had lost 43 per cent in weight.   In Cat 2 a crossing over is likewise noted on the 17th day, when the animal had lost 32 per cent in weight, but this cat also was killed after 28 days of fasting, at a time when its weight had diminished 48 per cent.   It is difficult to determine exactly when the crossing of the curves occurred in Cat 3, but on the 21st day this is very definite.   The cat was killed on the 26th day.   Cat 4 was killed after 14 days of fasting, when its loss in weight was slightly above 30 per cent, and the crossing of the curves can be seen the day before it was killed.   In Cat 5 the crossing is already present on the 5th day of fasting, when the animal has lost barely 12 per cent in weight, i.e. at a very early stage, and the two curves separated widely by the 10th day of fasting.   Finally, in Cat 6 the crossing is found on the 26th day, when the animal has lost 40 per cent; in other words, in an advanced stage of inanition.   Obviously, the crossing over of the creatine and creatinine excretion curves is a more general occurrence in cats than in dogs but, contrary to Palladin and Epelbaum, this certainly stands in no relationship to the actual stage of inanition nor is it at all a warning of the premortal condition of the experimental animal.   In the cat, as was the case in our dogs, the crossing over is a purely accidental occurrence, and in the dog it is apparently much less frequent than in the cat.   It is well to recall that Howe, Mattill, and Hawk's dog which fasted 117 days did not show at any time this crossing over, which the authors attributed to the fact that this dog, although it had already lost more than 60 per cent in weight, had not yet reached the terminal stage of inanition.

TABLE II.

| Dog No. | Period. | Change in body weight. | N partition in urine. | | | | Creatinine coefficient. |
|---|---|---|---|---|---|---|---|
| | | | Total N. | NH₃-N. | Creatinine N. | Creatine N. | |
| | | *per cent* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 7,  16.08 kilos. | 1. P, 3 days. | | 32.85 | 1241 | 617 | 827 | 12.7 |
| | 2. " 4 " | | 49.10 | 2267 | 743 | 918 | 11.4 |
| | 1. F, 3 days. | −5.5 | 11.79 | 416 | 497 | 206 | 10.4 |
| | 2. " 3 " | −9.8 | 10.53 | 452 | 476 | 228 | 10.4 |
| | 3. " 4 " | −14.9 | 13.06 | 545 | 632 | 233 | 10.9 |
| | 4. " 4 " | −20.7 | 12.95 | 563 | 577 | 137 | 10.7 |
| | 5. " 4 " | −24.4 | 12.23 | 506 | 615 | 211 | 12.1 |
| | 6. " 4 " | −28.8 | 11.34 | 470 | 542 | 194 | 11.2 |
| | 7. " 4 " | −32.8 | 11.65 | 397 | 535 | 228 | 12.6 |
| | 8. " 4 " | −37.7 | 11.65 | 412 | 432 | 190 | 10.1 |
| | 9. " 4 " | −40.4 | 5.76 | 155 | 268 | 136 | 6.7 |
| 9,  16.43 kilos. | 1. P, 4 days. | | 48.00 | 2058 | 915 | 396 | 13.8 |
| | 2. " 4 " | | 54.40 | 2232 | 923 | 493 | 13.7 |
| | 1. F, 4 days. | −5 7 | 12.98 | 622 | 588 | 166 | 11.9 |
| | 2. " 4 " | −11.8 | 16.13 | 396 | 770 | 201 | 12.4 |
| | 3. " 4 " | −18 0 | 17.60 | 581 | 533 | 405 | 9.2 |
| | 4. " 4 " | −23.2 | 21.05 | 869 | 554 | 308 | 10.3 |
| | 5. " 4 " | −29.3 | 21 20 | 772 | 643 | 408 | 12.9 |
| | 6. " 3 " | −33 2* | 13 56 | 300 | 335 | 519 | 9.6 |

Food consumed by Dogs 7 and 9:

| | Dog 7. | | Dog 9. | |
|---|---|---|---|---|
| | Preliminary period. | | Preliminary period. | |
| | 1 | 2 | 1 | 2 |
| Beef heart, *gm*.. .... ... ....... | 1440 | 1920 | 1520 | 1520 |
| Sucrose, *gm*................. ...... | 240 | 320 | 320 | 320 |
| Fat mixture, *gm*......  ..  ...... | 192 | 256 | 256 | 256 |
| Water, *cc*................. . ..... | 1680 | 1945 | 800 | 800 |

* The dog died accidentally while a sample of blood was being taken from the heart.  For the last few days the dog was sick, and on the 2nd day of the last fasting period showed albumin in the urine.

A study of the creatinine coefficient during fasting is also interesting.  In our experiments this either remains remarkably

constant throughout the larger part of the fast, or it may show a tendency to increase or to decrease. However, in the advanced stages of fasting, *i.e.* at the time when the loss in weight already exceeds 30 per cent, the coefficients may actually become very low. Thus, in Dog 2 the creatinine coefficient is practically the same as before the fast until the loss in weight is 31 per cent, when the coefficient suddenly drops to 4.6; in Dog 7 (Table II) the coefficient remains more or less constant until the loss in weight is 40 per cent, when it suddenly diminishes to 6.7; in Dog 9 the condition is the same except that the diminution of the creatinine coefficient is much less marked. Palladin and Epelbaum as well as Ssawron (9) report values for the creatinine coefficient during fasting which are not only very variable but also exceedingly high. However, these values need hardly be considered seriously at all since they are simply crude arithmetical errors.

Since nearly 98 per cent of the creatine of the organism is contained in its muscles, and the proportion of the skeletal muscles in the whole body is known, it is possible to compute how much creatinine could be derived from that source. In normal dogs the muscles make up practically 40 per cent of the body weight, and in fasted dogs about 33 per cent. It is easy, therefore, from the initial and final weights of the experimental dogs to determine how much muscle substance has been lost during the fast. Furthermore, dog skeletal muscles contain on an average 0.357 per cent creatine (5). From these data it can be shown that the total creatinine nitrogen excreted by each dog in the course of its fast exceeds by a greater or lesser amount that which could be derived from the entire muscle mass catabolized. In our eight fasting dogs this excess ranged from 1.37 to 4.30 gm. This discrepancy is the more striking because the total nitrogen excretion corresponds very well with that which could be accounted for as resulting from the loss of muscle tissue. In Howe, Mattill, and Hawk's dog which fasted 117 days the discrepancy between the actually found excretion of total creatinine and that which might be expected from the disintegrated muscle substance is still greater; namely, 10.73 gm. or more than half of the total excretion (20.4 gm.). Of course, we know that in fasting muscle the creatine content increases, and by using the factor 0.357 which was found in normal dog muscle an error is introduced

which tends to lower the result, thus giving less creatinine from the wasted muscle tissue than actually corresponds to the higher creatine content. However, the increase in muscle creatine is not more than perhaps 30 per cent of the normal value, whereas our calculations show that the amount unaccounted for on the basis of muscle catabolism is 25 to 50 per cent of the total creatinine excretion. A considerable part of the creatinine excretion must, therefore, come not from preformed creatine of the muscle but from actual synthesis. The increased creatine content of muscle of fasting animals would bear out this assumption.

Mendel and Rose (6) and Benedict and Osterberg (1) postulate that inanition leads to an increased creatine formation, a view with which we fully concur from the evidence of our experiments.

It might be supposed, of course, that the excessive quantity of total creatinine in the urine is derived from the creatine liberated in very advanced stages of fasting from the remaining muscle mass. This is possibly the explanation of the increased creatine appearing in the urine of some animals in the late stages. Howe and Hawk (3) at any rate found a considerable diminution in the muscle creatine in a dog after long fasting. Myers and Fine (7) reported a diminution in muscle creatine in rabbits after long fasting. Palladin and Epelbaum (8) likewise observed in their fasting cats during the fourth inanition period (loss in body weight 40 per cent or more) that the muscle creatine was greatly reduced. However, even then it was still above the prefasting concentration, while Chanutin and Silvette (2) show that in the rat the muscle creatine continues to rise even at a time when the animals have already suffered a loss in weight of 40 per cent.

The work of Chanutin and Silvette is also interesting because it suggests that creatinuria ordinarily observed in fasting animals cannot be attributed to a failure of the muscles to hold extra creatine, thus leading to its excretion through the kidneys. These authors demonstrate by feeding creatine that the tissues can actually take up appreciable quantities beyond their ordinary concentration. The excretion of the creatine cannot, therefore, result simply from supersaturation of the muscles but must, in some way, be associated with some general disturbance in the metabolism of the fasting organism. In view of our present knowledge, the greatly diminished carbohydrate metabolism dur-

ing fasting would seem the most probable cause of the creatinuria. However, it must be frankly admitted that a plausible explanation of the mechanism of fasting creatinuria is yet a hope rather than an accomplishment.

TABLE III.

| Dog No. | Period. | Change in body. weight. | N partition in urine. | | | | Creatinine coefficient. |
|---|---|---|---|---|---|---|---|
| | | | Total N. | NH₃-N. | Creatinine N. | Creatine N. | |
| | | per cent | gm. | mg. | mg. | mg. | |
| 10, 15.71 kilos. | 1. P, 4 days. | | 29.51 | 1407 | 614 | 178 | 9 9 |
| | 2. " 3 " | | 20.88 | 1532 | 484 | 188 | 10.4 |
| | 1. F, 4 days. | −5.3 | 15.32 | 1106 | 561 | 186 | 9.3 |
| | 2. " 4 " | −9.3 | 10.90 | 665 | 622 | 154 | 10.8 |
| | 3. " 4 " | −12.2 | 9.74 | 624 | 560 | 129 | 10.2 |
| | 4 " 4 " | −15.2 | 8 97 | 497 | 554 | 146 | 10.4 |
| | 5. " 4 " | −18.4 | 10.11 | 531 | 523 | 126 | 10.2 |
| | 6. " 4 " | −22.0 | 7.20 | * | 520 | 99 | 10.6 |
| | 7. " 4 " | −28.4 | 8.70 | | 484 | 95 | 10.4 |
| | 8. " 5 " | −32 0 | 9.33 | | 570 | 116 | 10.6 |
| | 9. " 4 " | −35.0 | 8.33 | | 420 | 84 | 10.7 |
| | 1. R, 4 days. | +9.4 | 17.60 | | 40 | 196 | 0.95 |
| | 2. " 4 " | +18.0 | 24 73 | | 528 | 205 | 12.0 |
| | 3. " 4 " | +24.9 | 29.00 | | 616 | 479 | 12 6 |

Food consumed by Dog 10:

| | Preliminary period. | | Refeeding period. | | |
|---|---|---|---|---|---|
| | 1 | 2 | 1 | 2 | 3 |
| Beef heart, gm ...... .... ........ | 1375 | 750 | 1000 | 1500 | 1500 |
| Sucrose, gm........................ | 319 | 174 | 232 | 348 | 348 |
| Fat mixture, gm.................. | 220 | 120 | 160 | 240 | 240 |
| Water, cc.......................... | 800 | 1000 | 1100 | 1200 | 1200 |

* Urine alkaline, through remainder of experiment, owing to a persistent cystitis.

If the creatine is a product of muscle catabolism, as seems very probable, one might expect to find that the per cent of total creatinine nitrogen excreted in the urine would be approximately the same as the ratio of creatine nitrogen to total nitrogen in the

muscle; in other words, about 4 per cent. During the preliminary period this is practically the case. But in the course of a prolonged fast, in seven of our dogs the total creatinine nitrogen varies from 5.3 to 6.7 per cent of the total nitrogen eliminated during the same time, and in the case of Howe, Mattill, and Hawk's dog this was 5.7 per cent of the total nitrogen excretion. Our Dog 11 is an exception in that its creatinine excretion was unusually large (8.2 per cent). Although these results do not seem to lend much support to the view that the entire creatinine nitrogen excreted

TABLE IV.

*Daily Nitrogen Excretion of Fasting Dogs.*

| Condition. | Initial weight. | Average daily N excretion. | Creatinine coefficient (average). | Per kilo loss in weight. | | |
|---|---|---|---|---|---|---|
| | | | | Excreted N. | Total creatinine N. | Per cent of total N. |
| | *kg.* | *gm.* | | *gm.* | *gm.* | |
| Fasting with water. | 12.85 | 1.98 | 8 4 | 17.0 | 1.13 | 6.7 |
| | 13.05 | 2 18 | 8 0 | 18.0 | 1.02 | 5.7 |
| | 15.40 | 2.40 | 10.8 | 16.7 | 1.12 | 6.7 |
| | 13.00 | 2.88 | 11.2 | 20.9 | 1 39 | 6.6 |
| | 20.00 | 2 92 | 11 8 | 17 5 | 1.43 | 8.9 |
| Fasting without water. | 16.24 | 2.97 | 10.6 | 15.7 | 0 99 | 5.8 |
| | 19 34 | 3.96 | 11.4 | 14.5 | 0.93 | 6.3 |
| | 16 70 | 4 46 | 11 0 | 19.1 | 1.02 | 5.3 |
| * | 26.33 | 3 08 | (8.4) | 21 8 | 1 23 | 5.6 |

* Results reported by Howe, Mattill, and Hawk on a dog which fasted 117 days.

during fasting is directly derived from the catabolized muscle substance, an examination of the data for each dog in the course of the fast shows that on the whole the ratio, varying for different dogs, is fairly constant for the individual dog, especially during the middle part of the fast. In three of our dogs this ratio of total creatinine nitrogen excreted to total nitrogen is about 5.5; in three other dogs the ratio is about 6.5. For Dogs 10 and 11 the ratios while also more or less constant are considerably higher. The constancy of the ratios is, of course, a strong argument in favor of the assumption that, even if the total creatinine excretion is

not directly related to the preformed creatine of the muscle substance, the nitrogenous metabolism determines the level of total creatinine (creatine and creatinine) elimination. The fact that the ratios are invariably higher than those expected if the creatinine was derived entirely from the preformed muscle creatine, argues further for the assumption that a new synthesis occurs during fasting, and in this connection it is also interesting to observe that the greater the total tissue catabolism, as measured by the total nitrogen excreted, the greater is the amount of total creatinine in the urine which cannot be accounted for on the basis of the preformed creatine of the wasted muscle mass.

Arranging the data for our eight fasting dogs according to the average daily nitrogen excretion, we obtain the results shown in Table IV. In general it seems from these data that the average daily excretion of nitrogen varies directly with the size of the dog and the creatinine coefficient likewise increases with the daily nitrogen elimination. It will also be observed that dogs fasting without water have a much greater daily nitrogen excretion than those receiving water. It is not, however, indicative of an increased nitrogenous metabolism, as may be seen from the fifth column which shows the number of gm. of nitrogen in the urine per kilo of loss in body weight. On the contrary, the dogs without water show a smaller waste of protein, and their total creatinine N elimination is likewise definitely less than in dogs fasting with water. In the latter 1.02 to 1.43 gm. of total creatinine were found in the urine for every kilo of loss in weight, while in the former the loss was only 0.93 to 1.02 gm. Since, however, the rate of loss of body weight is about $1\frac{1}{2}$ times as great in a fast without water as with water, we must conclude that under the first experimental condition fat must be much more extensively oxidized.

We must add a word of comment on the creatine-creatinine excretion during the refeeding periods. In Dog 3 the creatinine N gradually rises to the prefasting level, while the creatine N, remaining unchanged in the first refeeding period, quickly increases and even exceeds the normal after the third period. In Dog 5 the creatinine N excretion diminishes although the total N of the food and that of the urine increase during that time. The creatine N elimination is the same during all three refeeding periods and

is definitely low (0.55 per cent total N). In Dog 6 the creatinine N excretion is about the same as during the last two fasting periods, and about 50 per cent lower than during the preliminary period, though the same food, both quantitatively and qualitatively, was consumed. The creatine N excretion is only slightly higher than in the last few fasting periods, and considerably lower than during the preliminary period. In Dogs 10 and 11 both the creatine and creatinine N excretion increase progressively during the refeeding. However, in Dog 10 the creatinine N during the third refeeding period is the same as during the preliminary and the creatine N is much higher, while in Dog 11 both the creatine and creatinine N remain below the level found in the preliminary experiment. The thing that is particularly remarkable in the refeeding experiment with these two dogs is the fact that the creatinine N during the first refeeding period is extremely low so that the creatinine coefficient during those few days falls below 1. In four out of the five dogs which were being refed after a long fast there is thus unmistakable evidence of a retention of creatine.

### SUMMARY.

1. Experimental evidence is advanced to show that the "creatine-creatinine crossing" is not necessarily a phenomenon preceding death from fasting, but a peculiarity of the fasting metabolism.

2. The creatinine coefficient is generally very constant, even during a long fast, and not materially different from that found during feeding. However, in very advanced stages of inanition the coefficient may diminish more or less sharply.

3. The view-point of a synthesis of creatine during inanition receives support from these experiments.

4. During refeeding following a long fast there is evidence of a retention of creatine.

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# AN IMPROVED TECHNIQUE FOR MICRO CALCIUM ANALYSIS.

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During the course of some investigations on the combination of calcium with certain compounds of biological interest, it became necessary to analyze solutions containing from 1 to 5 mg. of calcium in the sample available for analysis. This is an amount for which the usual macro methods are not suitable and does not fall within the range of blood calcium. In dealing with these amounts it became necessary to find a method suitable for the separation of calcium oxalate precipitate, and for this we devised a micro filter which so improves the usual accuracy and speed of this procedure that it seemed desirable to test its applicability to still different situations. While it is possible to separate the precipitate by centrifuging, this method has certain disadvantages in that the precipitate has a tendency to creep up the sides of the tube and also to float on the surface of the liquid. We have been able to show, not only that this filter is advantageous for several mg. of calcium, but that it is very useful in the analysis of such amounts as are found in blood.

## EXPERIMENTAL.

Fig. 1 shows the design of the micro filter. On the square shoulder is placed a platinum plate cut from foil with a cork borer, and having small punched holes. It is from a quarter to three-eighths of an inch in diameter. In constructing the filter, it is desirable that the glass should not be constricted below the shoulder. The glass blowing consists simply in attaching a tube of about ¾ inch cross-section to one of about ⅜ inch cross-section, which in turn is attached to one of about 4 mm. cross-section.

This is slightly pushed together while hot, thus forming the shoulder. On the platinum plate is formed a thin asbestos pad made from well washed asbestos. The filtration is carried out under mild vacuum pressure.

For the analysis of calcium, the calcium oxalate is precipitated in the usual way, by addition of ammonium oxalate to the hot, slightly acid solution, with subsequent neutralization with ammonia, and standing for some hours. The precipitate is separated by filtration through the micro filters and is washed with dilute
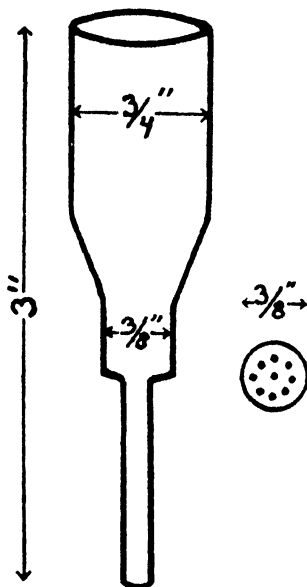


FIG. 1. Micro filter.

ammonia saturated with calcium oxalate. The entire pad is removed by inserting a small stirring rod at the bottom and rinsing down the loosened pad with hot water, and then with hot 2 N sulfuric acid. The pad is rinsed back into the original beaker in which precipitation was carried out, and the titration is carried out in the presence of the platinum plate and asbestos pad with potassium permanganate of the proper strength.

In the case of blood analysis, the procedure is somewhat modified. The method of precipitation is that of Kramer and Tisdall

(1), but instead of the usual centrifugation, the serum is filtered as described and washed with dilute ammonia saturated with calcium oxalate. Instead of transferring the entire pad as before, we may now, in view of the very small amount of calcium oxalate present, dissolve it *in situ* with sulfuric acid. For this

TABLE I.

*Typical Analyses of Calcium in Sera and Calcium Lactate Solutions.*

| Substances and amounts employed. | Ca | Deviation from mean. | Substances and amounts employed. | Ca | Deviation from mean. |
|---|---|---|---|---|---|
| | mg. per 100 cc. | per cent | | mg. per 100 cc. | per cent |
| Beef serum, 2 cc. samples. | 11.2 | +0.5 | Pig serum, 2 cc. samples with 2 mg. calcium per 100 cc. added. | 13.0 | −1.1 |
| | 11.2 | +0.5 | | 13.1 | −0.4 |
| | 11 1 | −0.5 | | 13.1 | −0.4 |
| | 11.2 | +0.5 | | 13.0 | −1.1 |
| | 11.1 | −0.5 | | 13.3 | +1.1 |
| | 11 1 | −0.5 | | 13.4 | +1.9 |
| Sheep serum, 2 cc. samples. | 10.9 | +3.5 | Calcium lactate stock solution, 25 cc. samples. | 40.24 | +0.06 |
| | 10.5 | −0.5 | | 40.16 | −0.14 |
| | 10 4 | −1.5 | | 40 28 | +0.16 |
| | 10.4 | −1.5 | | 40.28 | +0 16 |
| | | | | 40.16 | −0.14 |
| Sheep serum, 2 cc. samples with 2 mg. calcium per 100 cc. added. | 12.7 | +1.4 | | 40.16 | −0.14 |
| | 12.2 | −2.5 | | 40 24 | +0 06 |
| | 12.7 | +1.4 | | | |
| | 12 5 | −0.1 | Calcium lactate stock solution, 25 cc. samples with 16 mg. calcium per 100 cc. added. | 56 34 | 0 |
| Pig serum, 2 cc. samples. | 11.2 | −1 2 | | 56.40 | +0.07 |
| | 11.7 | +3.2 | | 56 40 | +0.07 |
| | 11.2 | −1.2 | | 56.24 | −0.24 |
| | 11.3 | −0.3 | | | |
| | 11.3 | −0.3 | | | |

purpose a battery of small suction flasks is set up. Each flask is of such a size that the centrifuge tubes in which precipitation is carried out will stand upright inside of the flask with the mouth around the lower end of the filter. The filter is simply transferred after the last washing to the small flask and 1 cc. of hot 2 N

sulfuric acid is used to rinse down the sides of the filter.   This is allowed to stand for some seconds and is then drawn through slowly with light suction.   This is repeated twice, making three washings of 1 cc. each.   This will quantitatively dissolve and transfer the precipitate back to its original tube, provided the washing is not hurried too much.   The solution may then be titrated as in the original method or it may be treated according to the gasometric method of Van Slyke and Sendroy (2).   In the results on blood shown in Table I, simple titration was employed. This procedure is less accurate than that of Van Slyke and Sendroy. On the other hand the process of filtration is probably more accurate than centrifugation.

It should be noted that when the sample is larger than the 0.2 mg. present in the above blood samples, the accuracy is correspondingly increased.   In our analysis using from 2 to 10 mg. of calcium in the sample, we have run several hundred determinations and find an average error less than 0.5 per cent.

## SUMMARY.

A micro filter is described, which facilitates the analysis for small amounts of calcium by the volumetric method, in that it increases the speed and accuracy of the determination and materially reduces the necessary amount of equipment.   The method is shown to be suitable for blood calcium analysis, as well as for slightly larger amounts than are found in blood samples.

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# A RAPID AND ACCURATE METHOD FOR DETERMINING THE QUANTITY OF YEAST OR OTHER MICRO-ORGANISMS IN A SUSPENSION.

By ROGER J. WILLIAMS, EDWARD D. McALISTER, AND RICHARD R. ROEHM.*

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In studies concerned with the nutrition of yeast it is desirable to have a method whereby one can determine yeast crops accurately and rapidly. The methods used by workers in recent years include (*a*) counting isolated colonies in hanging droplets (1, 2), (*b*) centrifuging the yeast in a calibrated tube (3–5), (*c*) counting the yeast cells with a hematocytometer (6, 7), (*d*) weighing the dried yeast crop in a Gooch crucible (8, 9), (*e*) estimating the turbidity of suspension in a special comparator (10).

The senior author in his studies has tried all of these methods in approximately the order listed, but none has been found highly satisfactory. The weighing method and the hematocytometer method can both be used with suitable accuracy but they are time-consuming and require technique which is rather exacting. We have not found the centrifuge method very satisfactory or accurate, owing to difficulties in obtaining uniform packing. It is not useful for very wide variations in yeast crops unless sets of tubes of different dimensions are used. The turbidity comparator designed by Peskett is quite rapid and has been used by us with considerable success. However, it has not in our hands proved to be ·susceptible of sufficient accuracy or to be sufficiently free from the personal element.

Recently we have tried out a new method which from the very first has proved to be so much superior to the other methods that we are describing it for the benefit of other workers. It can be used not only to estimate yeast crops but to standardize very dilute suspensions for seeding.

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It involves interposing the yeast suspension in a suitable cell between a 6 to 8 volts light and a specially prepared thermocouple and measuring the E.M.F. set up by the thermocouple. An inexpensive galvanometer is sufficiently sensitive to allow an accurate estimate of the amount of yeast in suspension. Slight
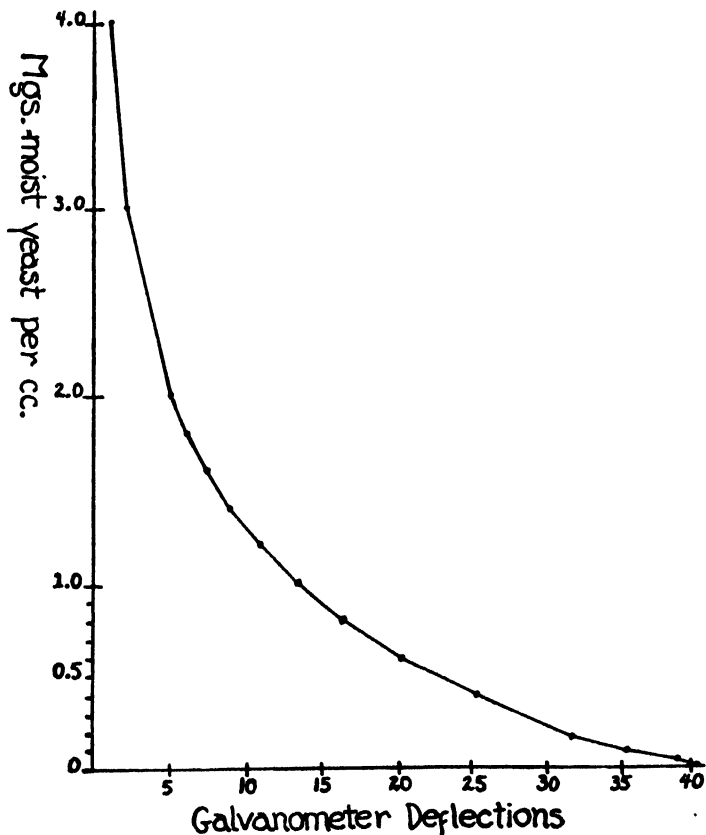
FIG. 1. Yeast in suspension *versus* galvanometer deflection (cm.).

differences in the composition of the interposed medium have practically no effect on the galvanometer deflection. Distilled water and the clear control medium which we have been using for yeast growth, when interposed in the same cell, gave practically identical galvanometer deflections.

Fig. 1 indicates the kind of results which may be obtained. A weighed quantity of starch-free bakers' yeast (69.5 per cent moisture) was suspended in 0.08 M sugar solution and fourteen different dilutions were made from the original. Each of the suspensions was interposed (with the same cell) and the galvanometer deflections recorded. By changing slightly the resistance in series with the lamp to compensate for the slow drop in the battery voltage, a constant deflection was maintained when a cell of distilled water was interposed. The galvanometer showed very little "zero creep" after the thermocouple had been illuminated for a short time.

After having established the curve, a series of five unknowns was prepared by one of us and tested by another, with the results

TABLE I.

*Determination of Amounts of Yeast in Suspensions.*

| Unknown sample No | Yeast present in 12 cc (dry weight) | Found. | Error. | |
|---|---|---|---|---|
| | *mg* | *mg* | *mg.* | *per cent* |
| 1 | 8 77 | 9 50 | +0.73 | 8.3 |
| 2 | 4 38 | 4 46 | +0.08 | 1.8 |
| 3 | 2 63 | 2 63 | 0.0 | 0.0 |
| 4 | 1 32 | 1 28 | −0.04 | 3.0 |
| 5 | 4 38 | 4 53 | +0.15 | 3.4 |

given in Table I. The first unknown was off the most desirable portion of the curve and showed a comparatively large error.

The apparatus used in this experiment was mounted as indicated in Fig. 2.

For this particular set of determinations a small cell about 3 × 4 cm. with about 2 cc. capacity was used. If it is desired to work with less turbid suspensions, a thicker cell can be used.

The thermocouple is similar to the type used by spectroscopists for intensity measurements (Fig. 3). It consists of a receiver (R) (upon which is focused a beam of light, the intensity of which is to be measured) and two small wires, one of bismuth the other an alloy of bismuth and tin (95.5 per cent Bi, 4.5 per cent Sn by weight). These wires are "spot welded" together and to the center of the receiver in one operation, and their other ends are welded (or soldered) to two relatively heavy copper leads. The

junction of these two wires with the receiver is the hot junction of the thermocouple, while their junctions with the two heavy copper leads constitute the cold junction.



Lamp    Lens    Cell    Lens    Thermocouple
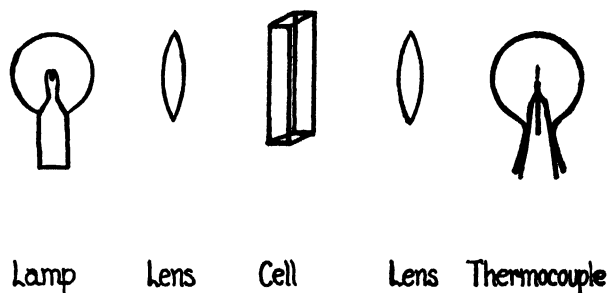
FIG. 2. Arrangement of apparatus.
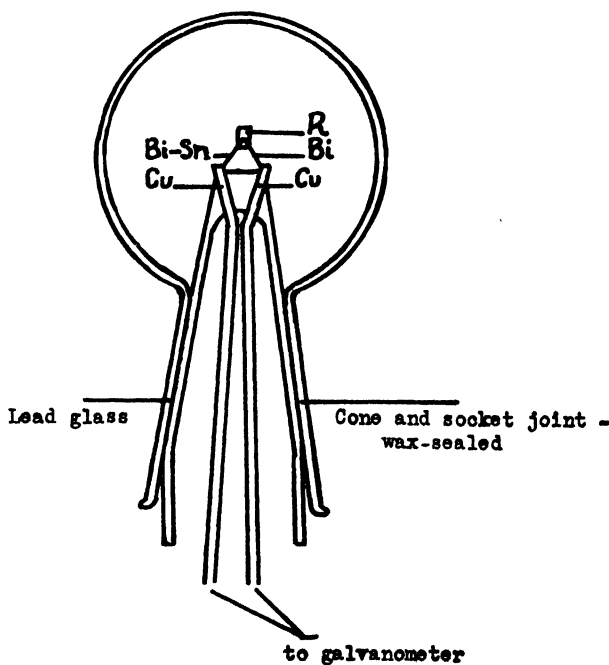


FIG. 3. Details of thermocouple.

For this type of work the receiver is made about 3 × 4 mm. of foil (tin or platinum, preferably the latter) about 0.003 mm. thick

and is blackened on the side exposed to the radiation. The bismuth and the alloy wires can be made by the Taylor process. In this process a small piece of the metal is inserted in a soft glass tube, that part of the tube next the metal is then heated to a dull red heat in a flame and quickly drawn out to the required diameter while out of the flame. The molten metal is thus drawn into a fine wire. The glass is then eaten off with hydrofluoric acid (5 to 15 minutes depending on the thickness of glass) and the wire then carefully freed from the acid.

In assembling the thermocouple it is essential that the two small wires be of the same length and diameter; otherwise there will be a serious zero creep. The couple should be inclosed in an air-tight container of glass (or one with a glass window) to eliminate troubles due to convection currents. Evacuation increases the sensitivity of the couple but is unnecessary for this type of work. Upon exposure to radiation the receiver reaches its equilibrium temperature (above that of the cold junction) in about $\frac{1}{8}$ of a second when in air at atmospheric pressure and when the couple is of the dimensions mentioned. Hence the response of the instrument depends only on the speed of the galvanometer used.

The method as outlined has the following advantages: (a) It is rapid. All of the dilutions and determinations here reported were completed within 2 or 3 hours. Furthermore this was carried out the first time this particular set of apparatus was assembled. A series of twenty yeast cultures can be evaluated easily within an hour. (b) It is accurate as is shown by the results given. We have confirmed its accuracy in other experiments not reported and have found it as accurate as is desired. The only important precaution is to have the yeast well suspended immediately before it is introduced into the cell. (c) It can be used over a very wide range of concentrations as is indicated by the curve. By varying the thickness of the cell the range can be almost indefinitely extended, except that very heavy suspensions settle too rapidly.

The only disadvantage for workers in general is the possible difficulties in building or procuring suitable thermocouples. The one used in this work was made by Dr. McAlister in the course of a few hours.

Aside from its use with yeast this apparatus can be used, without doubt, to determine the quantities of bacteria or other organ-

isms in suspensions. It can no doubt be used to determine quantitatively suspended matter of various kinds, and hence should be a very useful tool in the field of biochemistry.

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# THE EFFECT OF pH CONTROL IN THE AUTOCLAVING OF YEAST WITH RESPECT TO THE VITAMIN B FACTORS.

By ROBERT R. WILLIAMS, ROBERT E. WATERMAN, AND SAMUEL GURIN. ·

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Several investigators have described methods for the preparation of vitamin $B_1$[1] concentrates practically devoid of vitamin $B_2$.[2] However, very little has been reported on methods for procuring a rich source of vitamin $B_2$ free from vitamin $B_1$. Salmon, Guerrant, and Hays (1) have described a preparation, made from velvet bean leaves, relying upon the specificity of fullers' earth adsorption, solubility differences in alcohol, and heat. Smith and Hendrick (2), Sherman and Axtmayer (3), Chick and Roscoe (4), and other workers have relied upon autoclaved yeast to supply vitamin $B_2$, and their results seemed to justify the opinion that by this procedure yeast could be consistently freed of vitamin $B_1$ and still retain its vitamin $B_2$ potency. Results in our laboratory with the use of autoclaved yeast have been sufficiently irregular to justify investigation of this practice, especially since we felt that a satisfactory test for vitamin $B_1$ with rats necessitated a vitamin B-free diet plus a supply of vitamin $B_2$ entirely free from vitamin $B_1$.

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[1] Vitamin $B_1$ = The antineuritic vitamin which cures and prevents polyneuritis in rats and pigeons and which by itself is incapable of producing normal growth of rats on diets deficient in vitamin B.

[2] Vitamin $B_2$ = The factor which produces normal growth in rats when supplemented by adequate vitamin $B_1$. In this paper we are using the term vitamin $B_2$ in this somewhat loose sense, reserving for future discussion, certain evidence that vitamin $B_2$ in this sense is not a single entity. The pellagra-preventing factor is no doubt concerned but not necessarily to the exclusion of others.

## EXPERIMENTAL.

The rats used in this work were bred from stock maintained on Sherman's Diet 13 plus lean beef and were kept on this diet until 28 days old at which time they were put on experiment. The basal vitamin B-free diet (Sherman's Diet 107) consisted of:

|  | per cent |
|---|---|
| Extracted casein | 18 |
| ”　　 starch | 68 |
| Butter fat | 8 |
| Cod liver oil | 2 |
| Osborne and Mendel's salt mixture* | 4 |

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

We have found it essential to extract both the starch and casein thoroughly to remove variable but significant amounts of vitamin B factors. The method of extraction followed was that of Osborne and Mendel except that methyl alcohol at 50° was used as the solvent. We are by no means sure that even this diet is absolutely free of all vitamin B factors. The animals, when put on experiment, were placed in separate cages with raised bottoms to prevent coprophagy.

In order to determine "normal growth" with our strain of rats on this diet adequately supplemented by both vitamins $B_1$ and $B_2$, we fed 0.5 gm. of air-dried brewers' yeast daily.[3] Raising the dose to 1.0 gm. produced no better growth. This growth rate, however, was definitely greater than that given by Donaldson (5). This is especially striking in the males. Chart I shows some typical results. These higher growth rates may be due to a difference in the strain of rats used or very possibly to unrecognized deficiencies in the diets used in determining the Donaldson series of curves.

The method of autoclaving was as follows: Fresh brewers' yeast was spread out in shallow pans provided with covers and autoclaved for 6 hours at 15 pounds steam pressure in a sterilizer provided with an automatic pressure control. The yeast was then air-dried before a fan and ground.

[3] This yeast was furnished by the Jacob Ruppert Brewery, whose cooperation we heartily appreciate.

*Results.*

In our early work, with 1.0 gm. of autoclaved yeast daily, prepared by the above method, we were able to produce polyneuri-
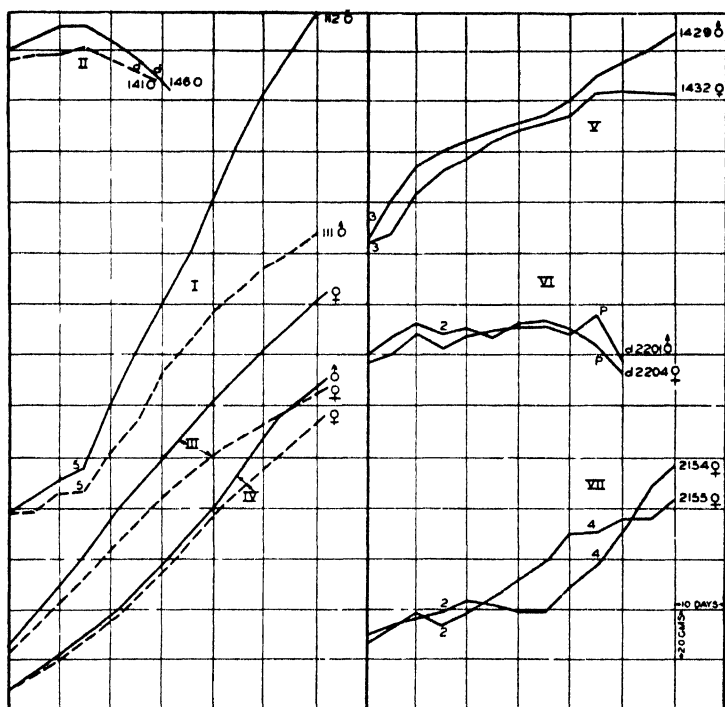


CHART I    Growth induced by brewers' *versus* bakers' yeast compared with accepted standards.    Curve I shows "normal growth" on our strain of rats with our basal diet supplemented by brewers' yeast    At point 5, 0.5 gm daily dose of brewers' yeast was added.    Curve II shows a typical curve of rats on the basal diet alone.    Curve III shows King's growth curves (from Donaldson's "The Rat").    Curve IV shows Donaldson's growth curves.    Curve V shows growth with bakers' yeast.    At point 3, 0 5 gm    daily dose of air-dried bakers' yeast was fed.    Curve VI shows growth with autoclaved bakers' yeast.    At point 2, 1 gm. daily dose of autoclaved bakers' yeast was fed.    Curve VII shows growth with auto-claved bakers' yeast supplemented with vitamin $B_1$.    20 mg. of activated fullers' earth were fed throughout and further supplemented at point 2 by 1 gm. of autoclaved bakers' yeast daily; at point 4, the autoclaved bakers' yeast dosage was increased to 2 gm.    P indicates polyneuritic symptoms; d, death.
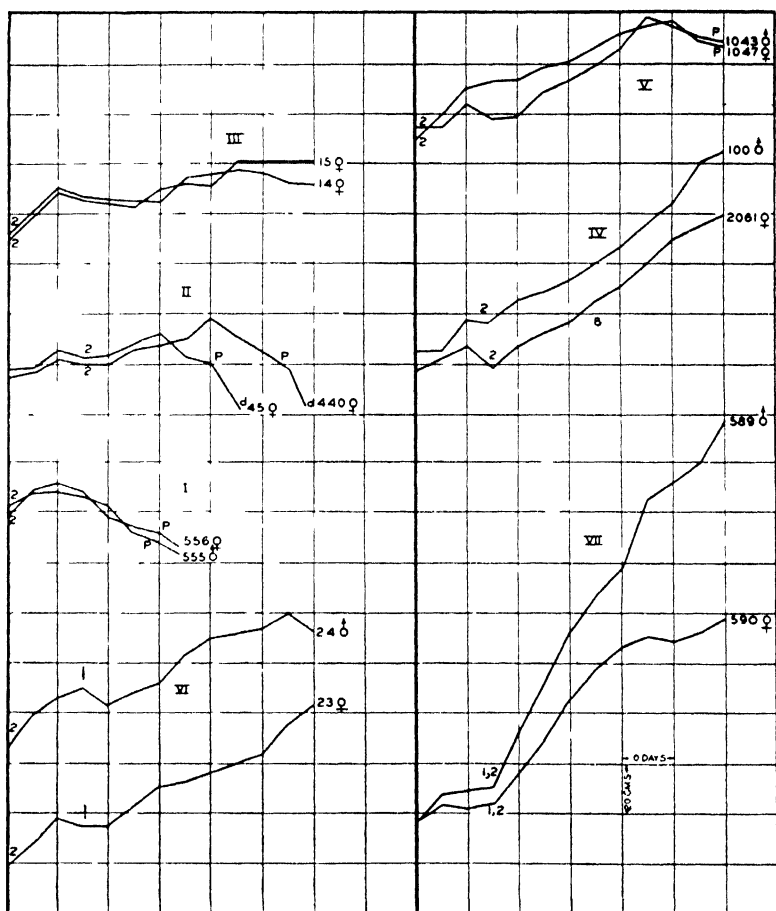
CHART II. This chart shows the irregularities in various lots of auto-claved yeast.   Curve I shows typical quick polyneuritis when full consump-tion of autoclaved yeast was not assured   Curves II and III show typical irregular results obtained with two lots of autoclaved yeast when fed mixed with the diet but not fully consumed.   Curve IV shows complete protection and some growth on an autoclaved yeast which was completely eaten. Curve V gives results with the same yeast as in Curve IV autoclaved for 12 hours and completely consumed   Curves VI and VII show variations in growth with different lots of autoclaved yeast supplemented by activated fullers' earth   At point 1, 20 mg. of activated fullers' earth were added; at point 2, 1 0 gm. of autoclaved yeast was added.   P indicates polyneuritic symptoms; d, death.

tis and death in 30 to 40 days. However, it was noticed that the rats rarely ate their full amount of addendum which was fed in a separate dish. When full consumption was encouraged by mixing the yeast with the diet, death in 60 days was seldom obtained and polyneuritic symptoms were rarely observed. Increasing the period of autoclaving to 12 hours produced some evidence of polyneuritis in 50 to 60 days but the rats in one group tested were able to survive the 60 day experimental period. These results (Chart II) indicated that there were still left varying but significant amounts of vitamin $B_1$ in our autoclaved yeasts.

The vitamin $B_2$ content of these autoclaved yeasts was also found to be variable when tested by supplementing with an adequate supply of vitamin $B_1$ which was furnished by a 20 mg. daily dose of an activated fullers' earth preparation previously found sufficient to prevent polyneuritis in both rats and pigeons (6). Under these conditions, variation in growth was observed with the different lots of autoclaved yeast in 1.0 gm. daily doses. Some of these autoclaved yeasts furnished almost normal growth, while others produced unmistakably subnormal results. These variations are also brought out in Chart II. There was a possibility that such differences might have been due to variations in vitamin $B_2$ content of the original fresh yeasts used. However, this possibility was ruled out when each of the fresh yeasts used was fed in 0.5 gm. daily doses and normal growth was obtained. We were thus forced to the conclusion that some of the vitamin $B_2$ in these yeasts had been destroyed during the process of autoclaving and that furthermore this rate of destruction varied with the different lots.

The possibility that these variations in vitamin $B_1$ and vitamin $B_2$ content might be a function of the hydrogen ion concentration was considered and the effect of varying the pH of the moist yeast before autoclaving was studied. Accordingly, a large batch of fresh brewers' yeast was divided into four lots as follows:

*Lot 1.*—This lot was brought to pH 1.0 to 2.0 with HCl.

*Lot 2.*—This lot was left at its natural pH (4.5).

*Lot 3.*—This lot was made mildly alkaline to pH 8 with NaOH.

*Lot 4.*—This lot was made strongly alkaline to pH 12 to 14 with NaOH.

These four portions were simultaneously autoclaved as pre-
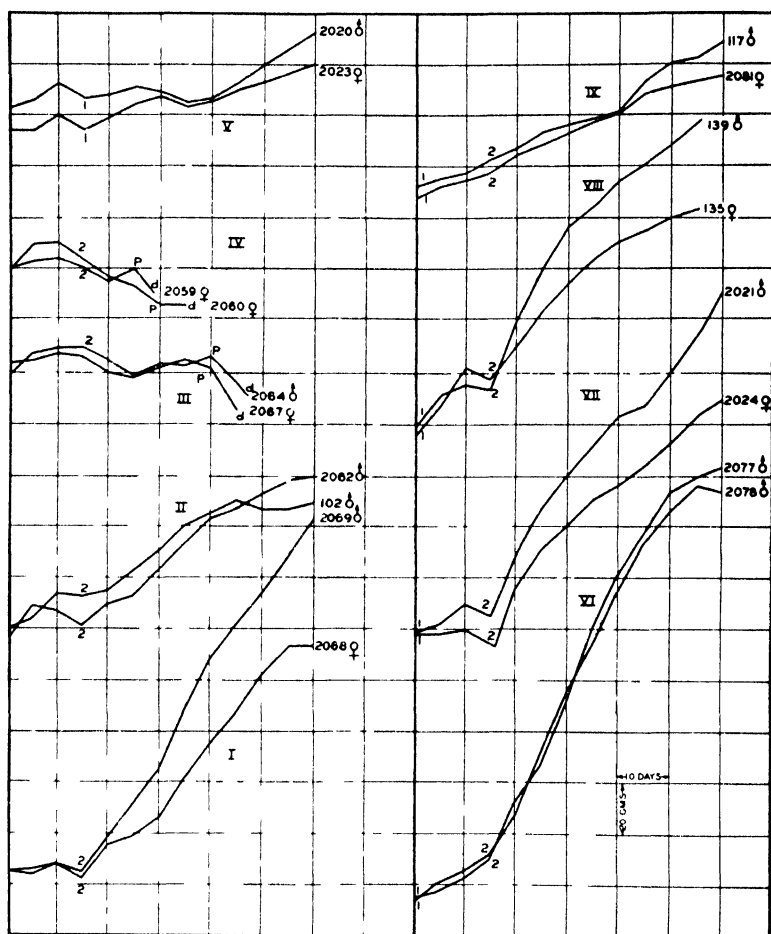
CHART III   Growth obtained with brewers' yeasts autoclaved at varying pH when fed as sole addendum to basal diet both alone and with further supplement of vitamin B₁   Curve I gives results on Lot 1; Curve II, Lot 2; Curve III, Lot 3; Curve IV, Lot 4; Curve V, 20 mg of activated fullers' earth alone; Curve VI, Lot 1 autoclaved yeast + 20 mg of activated fullers' earth; Curve VII, Lot 2 autoclaved yeast + 20 mg of activated fullers' earth; Curve VIII, Lot 3 autoclaved yeast + 20 mg of activated fullers' earth; Curve IX, Lot 4 autoclaved yeast + 20 mg. of activated fullers' earth   At point 1, 20 mg of activated fullers' earth were added; at point 2, 1 0 gm of autoclaved yeast was added   Full consumption of autoclaved yeast was assured   P indicates Polyneuritis: d, death.

viously indicated, neutralized as required with NaOH oɪ HCl, air-dried, and ground.                                        .

As a test for the presence or absence of vitamin B₁ these treated yeasts were fed in daily doses of 1.0 gm. as the sole addendum to the vitamin B-free diet.

Chart III brings out the following evidence.

*Lot 1.*—It will be seen that the rats were fully protected from polyneuritis and gained considerable weight. Evidently enough vitamin B₁, as well as vitamin B₂, remained undestroyed to support almost normal growth.

*Lot 2.*—The animals have been completely protected from polyneuritis but have gained considerably less weight, indicating that there has been some destruction of either vitamin B₁ or vitamin B₂ or possibly both.

*Lot 3.*—Polyneuritic symptoms are in evidence at the 40 to 45th day. Evidently most of the vitamin B₁ has been destroyed. No weight increase was obtained.

*Lot 4.*—The rats showed polyneuritic symptoms in 25 to 35 days. It was noted that the animals suffered from severe diarrhea.

These results indicate strongly that autoclaving in itself for 6 hours at 15 pounds pressure is not sufficient to destroy the antineuritic vitamin in brewers' yeast unless the material is treated at a pH in excess of 4.5.

Each lot was then evaluated for the amount of vitamin B₂ present. The animals were fed basal diet plus 20 mg. daily of an activated fullers' earth (previously tested) throughout the experiment. After 15 days, 1.0 gm. daily doses of these autoclaved yeasts were added as a further supplement with the following results (Chart III).

*Lot 1.*—The animals showed normal growth. Evidently a sufficient supply of vitamin B₂ was furnished.

*Lot 2.*—The growth obtained was good but definitely subnormal and was considerably less than that shown by the rats on Lot 1.

*Lot 3.*—The animals did not show as good growth as those on either Lot 1 or Lot 2. Considerable vitamin B₂ has been destroyed.

*Lot 4.*—Very slight growth was obtained. Only traces of vitamin B₂ were left undestroyed.

We interpret the series of experiments to mean that although vitamin B₂ is sufficiently more stable to heat than vitamin B₁, so

that its existence can be so demonstrated, nevertheless it is by no means entirely thermostable, especially in alkaline conditions. This conclusion seems justified by the fact that the symptoms included those of polyneuritis in the first series but not in the second in which vitamin $B_1$ was separately supplied.

In view of the difficulties encountered with brewers' yeast, and since autoclaved bakers' yeast is so extensively used as a source of vitamin $B_2$, an investigation of the latter was thought worth while. Air-dried bakers' yeast in 0.5 gm. daily doses was fed to rats as a sole addendum. The results, as shown in Chart I, indicate this material to be much less potent than brewers' yeast. When autoclaved in the usual manner, polyneuritic symptoms and death were brought about in the usual 30 to 40 days, but when supplemented by a rich source of vitamin $B_1$ complete growth was not obtained. Later experimentation has shown that if fed in somewhat larger doses (0.7 to 1.0 gm.) fresh bakers' yeast will produce normal growth in rats without further supplement. The poor growth obtained when 1.0 gm. of this autoclaved yeast was supplemented by adequate vitamin $B_1$ indicates again that there is destruction of vitamin $B_2$ in the autoclaving. These results are especially interesting, in view of the fact that it was found that the pH of the bakers' yeast used in this experiment was approximately 6.5. Various other lots of bakers' yeast have been tested by us for their acidity and the pH found to range from 5.5 to 6.5, while our brewers' yeast was consistently near pH 4.5. Such low acid concentration may very well account for the lack of vitamin B activity in commercial lots of dry yeast, especially if dried at relatively high temperatures.

<center>DISCUSSION.</center>

The results noted above strongly indicate that autoclaving brewers' yeast at 15 pounds steam pressure for 6 or even 12 hours does not insure complete destruction of the vitamin $B_1$ present. The resistance of vitamin $B_1$ to heat in strong acid was not surprising, but the fact that after 6 hours treatment at 15 pounds pressure in very weak acid (pH 4.5) there still remained considerable amounts of vitamin $B_1$ was striking. Alkaline autoclaving effectively destroys vitamin $B_1$. The persistence of vitamin $B_1$ activity in our autoclaved yeasts was not caused by poor heat

control since the autoclave was provided with an inside thermometer.

The complete removal of vitamin $B_1$ was always accompanied by serious losses of vitamin $B_2$ activity. At pH 8.0 this destruction is serious and is directly at variance with the belief that this factor is stable to alkali and heat. Hassan and Drummond (7) reported that vitamin $B_2$ was stable to heat and alkali, using however a much milder treatment than we have described. Chick and Roscoe (8) recognize the fact that destruction of vitamin $B_2$ takes place during heating. It is of interest to note that their yeast is washed with water before autoclaving, thus conceivably raising the pH.

The problem of procuring a rich source of vitamin $B_2$ free from vitamin $B_1$ still remains. It must be stressed that our purpose was to obtain normal growth. The dosages fed by certain other investigators have been considerably smaller than ours, due mainly to the fact that greater emphasis has been placed on the pellagra-preventive properties of the vitamin, rather than its growth-supplementing powers. It is probable that our larger doses have caused delay in onset of polyneuritis because of contamination with vitamin $B_1$, whereas this effect would be negligible in smaller amounts. Normal growth as we have determined it does not occur when these small pellagra-preventing doses are fed, properly supplemented with adequate vitamin $B_1$.

The discrepancy in stability towards heat and alkali between the pellagra-preventing factor and the growth-promoting factor suggests strongly that the two are dissimilar. In this connection the work of Palmer and Kennedy (9) and Hunt (10) is of possible significance, but further work is required to permit any conclusion.

### CONCLUSIONS.

1. The pH of yeast is an important factor in determining the efficiency of the destruction of the $B_1$ factor by autoclaving.

2. Autoclaving brewers' yeast at its natural acidity for 6 hours at 15 pounds pressure does not insure complete removal of vitamin $B_1$.

3. The $B_2$ factor is subject to destruction in the process of autoclaving unless carried out at high acid concentrations.

4. Alkaline autoclaving destroys a very large part of the $B_2$ factor originally present.

5. We have been unable to produce a vitamin $B_1$-free autoclaved yeast without involving considerable loss of vitamin $B_2$.

6. Autoclaving fresh bakers' yeast gives a product practically free from vitamin $B_1$ but low in vitamin $B_2$ content, possibly because of the relatively high pH of the material used.

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# THE BERGEIM TEST FOR INTESTINAL PUTREFACTION.

BY FREDERICK HOELZEL.

*(From the Department of Physiology of the University of Chicago, Chicago.)*

Bergeim (1924–25) suggested that the degree of reduction of ferric oxide in the digestive tract might serve as a measure of intestinal putrefaction. The present writer tried this test in personal experimentation but with apparently inconsistent results. This led to a critical examination of the method, in the course of which, among other things, it was found that similar degrees of reduction could be obtained whether the ferric oxide was ingested with the food or added to plain feces obtained with a similar diet. This seemed to indicate that the reduction of the ferric oxide occurred in the test-tube, instead of in the intestine as Bergeim supposed.

The cause of the reduction of the ferric oxide by fecal material (that is, when the mixture is heated with HCl to dissolve the iron) was not determined but it was still believed that products of putrefaction or fermentation were responsible. Some foods (glucose, lactose, wheat dextrin, powered egg albumin, and cellulose (Cellu Flour)) caused no reduction *in vitro*. Meat caused less than 5 per cent reduction. Coarse (undigested) food residues, separated from human feces by sieving and washing, caused little reduction. Recently, however, as a result of using the Bergeim test in attempting to estimate the degree of putrefaction in various segments of the digestive tract of rabbits, it was found that the food of the rabbits caused almost as much reduction as the feces. The food was a mixture of freshly‑ground carrots, oats, and alfalfa. The alfalfa proved to be responsible for most of the reduction and yielded higher figures than the mixture. In fact, with somewhat prolonged boiling of alfalfa and ferric oxide with HCl (5 to 8 minutes instead of the usual 2 minutes) as high as 85 per cent reduction would occur. Rabbit feces from the food mixture showed from 30 to 50 per cent reduction.

The high reduction due to alfalfa naturally suggested an explanation for the otherwise surprisingly high reduction (85 per cent) reported by Bergeim (1924–25, Table IV) when browned bread was the chief item in the experimental diet. *In vitro* tests made by the writer with plain bread and bread browned to various degrees showed respectively increasing degrees of reduction of ferric oxide up to over 50 per cent. Likewise, coffee (ground roasted beans) caused 55 per cent reduction. Evidently, heating, toasting, or roasting gives rise to at least one substance that reduces ferric oxide under the conditions by which the amount of reduction is determined.

### CONCLUSION.

The Bergeim test for intestinal putrefaction is not specific as high figures for reduction of ferric oxide are obtainable independent of any putrefaction.

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# BIOCHEMICAL FINDINGS IN A RARE CASE OF ACUTE YELLOW ATROPHY OF THE LIVER.

## WITH PARTICULAR REFERENCE TO THE ORIGIN OF UREA IN THE BODY.

By I. M. RABINOWITCH.

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Complete destruction of the liver in man, corresponding to total removal of the organ in the animal, must be extremely rare. As far as the writer is aware, there are no chemical records in the literature to indicate such a condition. It is because of this that the following case is reported.

The patient was a female, 28 years of age, who was admitted to the Montreal General Hospital on April 11, (Hospital No. 2022/29) and died on April 13, 1929. The characteristic clinical features of the disease were present; namely, jaundice, coma, and marked diminution of the area of liver dullness. During the postmortem examination Dr. L. J. Rhea, our pathologist, reported that the liver weighed 650 gm. Its normal markings were *entirely* absent and, on gross examination, it looked as though all of the glandular epithelium had disappeared leaving the framework only. Microscopically, this impression was proved to be correct, since, in spite of the many sections examined, only isolated liver cells were recognized and the staining properties of these remaining cells were poor. The kidneys showed acute degenerative nephritis, with marked fatty degeneration of the tubules. The anatomical changes here are significant, since, when combined with the functional changes to be discussed presently, the picture closely approximated the experimental removal of kidneys in animals.

There was no history of exposure to the chemicals to which the disease may be attributed, such as arsenic, phosphorus, chloroform, trinitrotoluene, etc. There was no pregnancy, etc. The condi-

tion may, therefore, be looked upon as, for the want of a better term, idiopathic acute yellow atrophy.

### Biochemical Data.

*Urine.*--Very little urine was obtainable. This fits in with the pathologist's report about the kidneys. The little which was obtained was very dilute. Its specific gravity was 1.004. The concentration of total nitrogen, determined by the Kjeldahl method, was 0.110 per cent, as compared with a normal concentration which is about 1 per cent. The concentrations of urea, amino acid, and ammonia nitrogen were 0.07, 0.07, and 0.063 per cent respectively. Because of the finding of such low concentrations of the substances mentioned above, the tests were repeated and all of the analyses were made in triplicate. The results were the same.

The urea was determined by the Van Slyke and Cullen method, the amino acid nitrogen by the Folin (1922) procedure, and the ammonia nitrogen by the Folin aeration method.

*Blood.*— The blood sugar was 0.046 per cent before, and 0.030 per cent after fermentation. The method employed in these determinations was that previously reported (1). There were thus only 16 mg. of reducing substances per 100 cc. of blood, which could be attributed to fermentable sugar, which presumably was glucose. There was, therefore, marked hypoglycemia, which, again, fits in with the result of complete removal of the liver in animals.

There was no urea in the blood. As far as the writer is aware, there is no such record in the literature. This finding was sufficiently striking to demand repetition of the test. The test was repeated four times with similar results. It need hardly be remarked that all possible technical details were checked and possible sources of error excluded, including the estimation of the efficiency of the urease. Added urea was recovered quantitatively.

The amino acid nitrogen content was 216 mg. per 100 cc. Folin's (1922) method was used. Because of the large quantity of nitrogen unexpectedly encountered, the test had to be repeated several times. The blood filtrate was finally diluted fifty times with water before the reading of the unknown was comparable with the standard.

The other data obtained with regard to the blood, though of little interest here, were as follows:

Plasma chlorides (as NaCl) .............. 0.550 per cent.
    "     cholesterol........................ 0.166  "    "
Inorganic phosphorus ................... 8.75  mg. per 100 cc.
Calcium....... ......... ................ 9.71  "   "   "   "
Creatinine............................... 1.30  "   "   "   "
Uric acid................................ 1.96  "   "   "   "

## *Discussion of Results.*

The very dilute urine and the anatomical changes found in the kidney, fit in with practically complete suppression of urinary function.   In spite of this there was no urea in the blood.   These findings are similar to those obtained by Bollman, Mann, and Magath (2) who not only removed the livers from animals but the kidneys as well.   The resultant anuria did not lead to accumulation of urea in the blood.   The findings in this rare case of acute yellow. atrophy, therefore, support the view that urea is formed exclusively in the liver.

As stated before the liver weighed 650 gm.   Its water content was 81.5 per cent.

The writer is indebted to Dr. A. T. Bazin, into whose service the patient was admitted and to Dr. L. J. Rhea, our pathologist, for their cooperation.

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# AN ELECTROMETRIC METHOD FOR THE DETERMINA-
## TION OF CHLORIDES IN WHOLE BLOOD AND
## ANIMAL TISSUES.

By J. C. FORBES and HAZELWOOD IRVING.

*(From the Departments of Medicine and Biochemistry, Medical College of Virginia, Richmond.)*

A number of methods have been described for the determination of chlorides in blood and body tissues. Those which are generally used in clinical laboratories are based on the Volhard method, which essentially consists in adding an excess of silver nitrate to the solution and titrating the excess with standard thiocyanate in the presence of nitric acid and with ferric alum as indicator. The end-point is taken as the appearance of a salmon-red color due to the formation of ferric thiocyanate. Although these methods give quite reliable results, nevertheless, unless different investigators will exactly agree as to what the term salmon-red absolutely represents they will obtain different absolute results although these agree relatively.

Bond and Haag (1) in 1926 described an electrometric titration method for the determination of chlorides in blood plasma, serum, and Folin and Wu (2) blood filtrates. The method is accurate and very rapid, but it cannot be applied to the analysis of whole blood as some of the blood constituents, likely the hemoglobin, interfere with the end-point. The object of this investigation was to determine if the method could be modified so that it could be applied to the determination of chlorides in whole blood and also in body tissues. After various experimentations the following method has been developed and found to be satisfactory from the standpoint of accuracy as well as speed.

## Apparatus.

*Galvanometer.*—A portable d'Arsonval galvanometer, type 2320d, manufactured by Leeds and Northrup, Philadelphia, was used

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throughout. The ballistic on the enclosed lamp and scale type may be used if greater sensitiveness is desired. The type used is accurate enough for any ordinary work as it will give a deflection with about 0.01 cc. of a sodium chloride solution containing 1 mg., of sodium chloride per cc. of solution.

*Calomel Cell.*—A Clark calomel electrode vessel was filled in exactly the same way as for hydrogen ion determination except that a molar potassium nitrate solution saturated with calomel was used instead of the usual potassium chloride-calomel solution. Potassium chloride cannot be used in this case since the diffusion of the chlorides into the titrating vessel would lead to erroneous results. Potassium nitrate was therefore substituted as it can be readily obtained in a pure form free from chlorides.

*Silver Electrode.*—This electrode may be prepared from 14 or 18 gage pure silver wire.or if desired it may be fused to a piece of pure silver sheet so as to expose a greater surface to the solution. The electrode must be kept clean. The authors experience has been that rubbing the electrode thoroughly with moist sodium bicarbonate and then thoroughly washing with water is the most convenient method for doing this.

In arranging the apparatus one terminal of the galvanometer is connected to the calomel cell and the other to the silver electrode. The calomel electrode is connected to the titrating vessel by an agar-saturated ammonium nitrate or an agar-normal potassium nitrate bridge. A simple spring is interposed anywhere in the circuit and is given a quick tap whenever it is desirable to note deflection of the galvanometer. Fig. 1 illustrates the general hook-up.

### Preparation of Solutions.

*Silver Nitrate Solution.*—2.905 gm. of c.p. silver nitrate are dissolved in a small amount of distilled water and transferred to a liter volumetric flask. 500 cc. of concentrated nitric acid are then added and the solution made up to volume with distilled water. 1 cc. of this solution is equal to 1 mg. of sodium chloride. Since nitric acid often contains a small amount of chlorides it is advisable to standardize the silver nitrate solution gravimetrically when great accuracy is required.

*Sodium Chloride Solution.*—This solution must be standardized

against the silver nitrate solution so it need not be very accurately prepared. It should contain approximately 1 mg. of sodium chloride per cc. The sulfuric acid, nitric acid, and ammonium hydroxide used must be C.P. chemicals and should be as free from chlorides as possible.
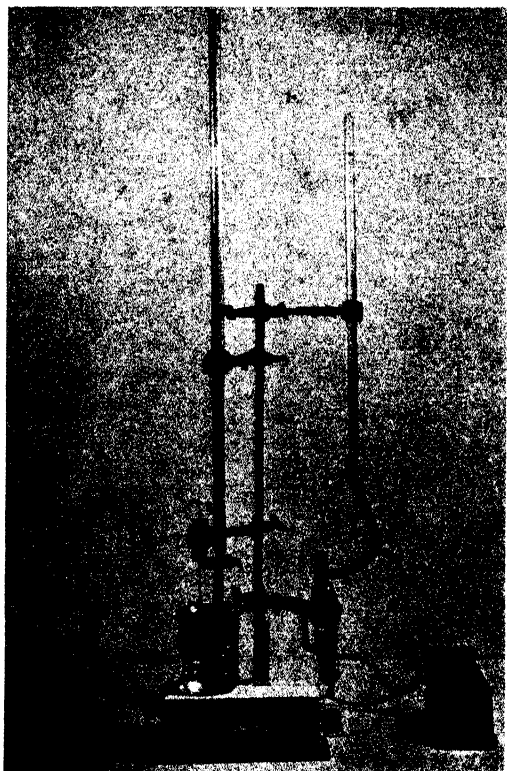


FIG. 1. The general hook-up of the apparatus showing the connection of one terminal of the galvanometer, at the right, to the calomel electrode vessel fitted with a M KNO₃ solution saturated with HgCl. On the left are shown the column of NaCl, the silver electrode, and the key.

## Standardization of Sodium Chloride Solution.

10 cc. of the standard silver nitrate were placed in a tall 250 cc. beaker, 1 cc. of concentrated sulfuric acid added, and the solu-

tion diluted with about 2 volumes of distilled water.    A few drops
of methyl orange were added as indicator and ammonium hydrox-
ide added to alkaline reaction, after which 0.1 cc. of concentrated
sulfuric acid was added so as to make the solution again distinctly
acid.    The solution was then cooled and titrated with the sodium
chloride solution until the galvanometer indicated zero deflection.
In any series of experiments it is very important that the volume of
the solutions at the conclusion of the titration be as near the same
as possible since the end-point is slightly affected by the total
volume.    A final volume of approximately 75 cc. was found very
convenient.    Blank determinations were made on the solutions
and this correction either added to or subtracted from the origi-
nal titration depending on whether silver nitrate or sodium chloride
was required.    The slight variations obtained in the blank from
time to time were very likely due to traces of chloride impurities
in some of the distilled water.    The values for these blanks varied
from 0.07 cc. of $AgNO_3$ to 0.12 cc. of NaCl.    It is advisable to
run blanks and restandardize the sodium chloride solution fre-
quently in order to eliminate any error due to improperly washed
electrode or impurities in the solutions used.

### Determination of Chlorides in Potassium Chloride.

A solution of c.p. potassium chloride was made up and analyzed
gravimetrically for chlorides (Table I).    It contained 3.040 mg.
of chlorine per 5 cc. of solution.    5 cc. of this solution were placed
in each of several 250 cc. beakers, about 20 cc. of water added, and
10 cc. of the standard silver nitrate solution added with constant
stirring.    Sulfuric acid and ammonium hydroxide were added as
in the standardization of the sodium chloride solution and the
excess silver nitrate titrated in the manner already described.    The
titration value was then corrected for blank determination and
from this corrected value mg. of chlorine per 5 cc. of potassium
chloride solution was calculated.

### Determination of Chlorides in Blood.

In the majority of these series of analyses 10 cc. of blood were
transferred to a 100 cc. volumetric flask, the pipette rinsed with
water, and the solution diluted to volume.    10 cc. of this diluted
blood were then transferred to large Pyrex test-tubes for digestion.

Equally satisfactory results may be obtained by adding 1 cc. of blood to 8 or 9 cc. of water in a test-tube and rinsing the pipette several times with the solution. This latter method is recommended for clinical use. 10 cc. of the standard silver nitrate were then added with stirring to each tube, 1 cc. of concentrated sulfuric acid added, and the tubes placed in water and kept at almost boiling temperature on a hot plate for about 2 hours. They were then cooled, transferred carefully to 250 cc. beakers, concentrated ammonium hydroxide was added until the yellow color of the solution turned slightly orange, indicating a slightly alkaline reaction, and then 0 1 cc. of concentrated sulfuric acid was added so as to

TABLE I

*Analysis of KCl Solution of Known Concentration*

| Series No | Determina-tion No | Chlorine added | Chlorine recovered | Error | Per cent error. |
|:---:|:---:|:---:|:---:|:---:|:---:|
|  |  | *mg* | *mg* | *mg* |  |
| 1 | 1 | 3 040 | 3 042 | +0 002 | +0 07 |
|  | 2 | 3 040 | 3 037 | −0 003 | −0 10 |
|  | 3 | 3 040 | 3 037 | −0 003 | −0 10 |
|  | 4 | 3 040 | 3 048 | +0 008 | +0 27 |
|  | 5 | 3 040 | 3 030 | −0 010 | −0 35 |
|  | 6 | 3 040 | 3 024 | −0 016 | −0 53 |
|  | 7 | 3 040 | 3 042 | +0 002 | +0 07 |
| 2 | 1 | 3 040 | 3 037 | −0 003 | −0.10 |
|  | 2 | 3 040 | 3 025 | −0 015 | −0 50 |
|  | 3 | 3 040 | 3 025 | −0 015 | −0 50 |
|  | 4 | 3 040 | 3 037 | −0 003 | −0 10 |

make the solution again distinctly acid. The solutions were then cooled and the excess silver nitrate titrated with sodium chloride in the usual manner. In Table II the results are compared with those obtained by Van Slyke's (3) method on the same blood.

*Determination of Known Chlorides in Dialyzed Blood.*

In order further to test the accuracy of the method, blood was laked with water, a little thymol added as preservative, and the whole dialyzed against tap water for 2 days and then for 2 days against distilled water. No chlorides could be detected in the

dialyzing fluid at the end of the dialysis. About 45 cc. of this solution were transferred to a 100 cc. volumetric flask and 50 cc.

TABLE II.

*Determination of Blood Chlorides.*

| Blood used. | Determination No. | NaCl per 100 cc. blood. Electrometric method. | NaCl per 100 cc. blood. Van Slyke method. |
|---|---|---|---|
| | | mg. | mg. |
| Beef. | 1 | 454 | 469 |
| | 2 | 453 | 468 |
| | 3 | 453 | 469 |
| | 4 | 450 | 468 |
| Average.. ...... | | 452 | 468 |
| Beef. | 1 | 497 | 501 |
| | 2 | 505 | 503 |
| | 3 | 500 | 495 |
| | 4 | 500 | 497 |
| | 5 | 500 | |
| Average ........ | | 501 | 499 |
| Human. | 1 | 480 | 473 |
| | 2 | 480 | 469 |
| | 3 | 486 | 474 |
| Average ........ | | 482 | 472 |
| Human. | 1 | 475 | 494 |
| | 2 | 476 | 482 |
| | 3 | 475 | 481 |
| | 4 | 486 | 481 |
| | 5 | | 476 |
| Average ........ | | 478 | 483 |

| Blood used. | Determination No. | NaCl per 100 cc. blood. Electrometric method. | NaCl per 100 cc. blood. Van Slyke method. |
|---|---|---|---|
| | | mg. | mg. |
| Human.* | 1 | 444 | 438 |
| | 2 | 434 | 437 |
| | 3 | ˙449 | 436 |
| | 4 | 441 | 445 |
| Average........ | | 442 | 439 |
| Rabbit | 1 | 506 | 501 |
| | 2 | 509 | 499 |
| | 3 | 511 | 500 |
| | 4 | 501 | 503 |
| Average. ....... | | 504 | 501 |
| Rabbit. | 1 | 512 | 501 |
| | 2 | 516 | 500 |
| | 3 | 519 | 503 |
| | 4 | 514 | |
| | 5 | 510 | |
| | 6 | 506 | |
| | 7 | 505 | |
| Average........ | | 512 | 501 |
| Rabbit. | 1 | 528 | 514 |
| | 2 | 527 | 509 |
| | 3 | 524 | 516 |
| | 4 | 526 | 513 |
| Average........ | | 526 | 513 |

* Analyses were made on mixed blood from which some plasma had been already removed in routine blood analysis. This is responsible for the low results.

of a potassium chloride solution containing 3.040 mg. of chlorine per 5 cc. added. The solution was then diluted to volume,

thoroughly mixed, and 10 cc. taken for analysis in the usual manner. The following figures give the result of this determination.

| Determination No | Chlorine added. | Chlorine recovered | Error. | Error. |
|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *per cent* |
| 1 | 3 040 | 3 024 | 0 016 | 0 5 |
| 2 | 3 040 | 3 012 | 0 028 | 0 9 |

### Determination of Chlorides in Animal Tissues.

Fresh liver was cut into fairly fine pieces with scissors and weighed amounts were transferred to the digestion tubes. 20 cc. of the silver nitrate solution and 1 cc. of concentrated sulfuric acid were then added and the material digested until the solution became light yellow in color. The excess silver nitrate was then titrated in the usual manner. The results of this determination are as follows:

| Determination No | Weight of tissue | Weight of NaCl. | Calculated weight of NaCl per 100 gm. tissue. |
|---|---|---|---|
| | *gm.* | *mg.* | *mg.* |
| 1 | 2 80 | 3 30 | 118 |
| 2 | 1 65 | 2 04 | 124 |
| 3 | 2 20 | 2 73 | 124 |

TABLE III.

*Comparison of Determinations Made by Authors' Method and That of Bond.*

| Series No | Determination No. | NaCl per 100 cc. blood. | |
|---|---|---|---|
| | | Authors' method. | Bond method. |
| | | *mg* | *mg.* |
| 1 | 1 | 458 | 457 |
| | 2 | 461 | 467 |
| | 3 | 458 | 462 |
| | 4 | 455 | 458 |
| Average | . . . . . . . . . . . | 458 | 461 |
| 2 | 1 | 432 | 427 |
| | 2 | 432 | 426 |
| | 3 | 432 | 424 |
| Average | . . . . . . . . . . . . . . . . . . . . . . . . . | 432 | 426 |

*Comparison of Authors' Method with Bond's Electrometric Method for Blood Filtrates.*

To eliminate errors of measurements as much as possible 10 cc. of mixed human blood were transferred to a 100 cc. volumetric flask, water added, and the pipette rinsed several times with this solution. 10 cc. of this diluted blood were taken for each analysis by the authors' method. The blood filtrate for Bond and Haag's method was prepared, as directed by them, by the Folin and Wu method; the first few cc. of filtrate were discarded and the remainder taken for analysis (Table III).

### DISCUSSION.

The chief advantage which the method here described has over the older methods for determining chlorides in whole blood and body tissues is that the end-point is more definite. It reduces to a minimum the personal equation which is always a factor when one is considering depth of color as is the case in the usual end-points. The apparatus required is inexpensive and may also be used for the direct titration of chlorides in blood filtrates, serum, or plasma by Bond and Haag's method. The technique is simple and consequently is well adapted for the routine determination of chlorides in whole blood or animal tissues.

### SUMMARY.

An electrometric method for the determination of chlorides in whole blood and animal tissues has been described. It has been found to give satisfactory results when tested out on solutions of known chloride concentrations as well as when compared with Van Slyke's method for the determination of chlorides in whole blood and tissues and with Bond and Haag's method for the determination of chlorides in Folin and Wu blood filtrates.

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# THE ISOLATION OF 3, 5-DIIODOTYROSINE FROM THE THYROID.

BY G. L. FOSTER.

*(From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York.)*

Harington and Randall (1) have recently reported the isolation of diiodotyrosine from the thyroid gland. In consideration of the yields obtained (yield not stated in their abstract) and in view of the losses entailed in the rather lengthy procedure, Harington and Randall conclude that diiodotyrosine and thyroxine are probably the only iodine compounds in the thyroid.

This paper reports the isolation of diiodotyrosine from partially purified thyroglobulin in yields sufficient to account for about 30 per cent of the total iodine. The process of isolation is based on the slight solubility of the lead salt of diiodotyrosine and the use of phosphotungstic acid to remove interfering substances. For, although Oswald (2) used phosphotungstic acid to precipitate diiodotyrosine, we have found that from dilute solution (400 mg. of total iodine in 2 liters) this reagent precipitates only about 5 per cent of the iodine.

The following description illustrates the method.

100 gm. of thyroglobulin (760 mg. of total iodine), 400 gm. of crystallized barium hydroxide, and 1000 cc. of water were boiled under a reflux for 18 hours and filtered while hot. The insoluble material contained 36 mg. of iodine. The filtrate was chilled and the barium hydroxide crystals filtered off and washed with cold water. The filtrate, which contained 720 mg. of total iodine and 85 mg. of iodine as iodide, was strongly acidified with glacial acetic acid (50 cc. beyond the maximum red color of methyl red). A small amount of dark colored precipitate formed. After 2 days in the ice box this precipitate was collected and was found to contain 212 mg. of iodine.[1] 185 mg. of pure thyroxine were iso-

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[1] The iodine content of this precipitate and the yields of thyroxine were highly variable in different experiments. The yield reported above is the highest we have obtained. Further work is in progress.

lated from the precipitate by crystallizing the sodium salt from dilute sodium carbonate, then precipitating from alkaline alcohol with acetic acid, as described by Harington (3). (It was identified by the color test with nitrous acid and ammonia, the melting point, and the iodine content.) To the filtrate were added 100 gm. of lead acetate in a small amount of water and the solution was filtered from lead sulfide. Ammonia was added to the filtrate till alkaline to phenolphthalein. 105 mg. of iodine remained unprecipitated. The precipitate was collected and washed well by grinding with 0.1 per cent ammonia. It was then suspended in water and acidified with glacial acetic acid, the small amount of insoluble material being removed by filtration. Dilute sulfuric acid was added till acid to Congo red and the lead sulfate was filtered off. To the filtrate were added 10 gm. of phosphotungstic acid in concentrated solution and the precipitate removed by filtration. Phosphotungstic and sulfuric acids were removed by barium hydroxide in slight excess. The solution was then acidified with acetic acid, 50 gm. of lead acetate added, and the lead salt precipitated by addition of ammonia as before. The precipitate was collected and washed thoroughly with water and was decomposed with hydrogen sulfide. The filtrate from lead sulfide was evaporated under reduced pressure to about 15 cc. and placed in the cold. The resulting crystals without purification weighed 0.440 gm. and contained 248 mg. of iodine. Dissolved in water with the help of a few drops of ammonia and reprecipitated with acetic acid, the well formed, whetstone-shaped crystals containing 58.3 per cent of iodine, melted at 197° when heated slowly and at 203° when heated rapidly.

### SUMMARY.

From a sample of 100 gm. of thyroglobulin (760 mg. of iodine) 33 per cent of the total iodine was isolated as diiodotyrosine and 16 per cent as thyroxine.

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# A RAPID AND ACCURATE METHOD FOR THE DETERMINATION OF UREA IN BLOOD.

By S. L. LEIBOFF and BERNARD S. KAHN.

*(From the Biochemical Laboratory of Lebanon Hospital, New York.)*

The theory upon which most of the present methods for the determination of urea nitrogen in blood are based, is the hydrolysis of urea by means of the enzyme urease. This procedure is the result of investigations made by Marshall (1), Van Slyke and Cullen (2), and Folin and Denis (3). After the urea has been converted into ammonium carbonate, various means are used to determine the nitrogen content. The ammonia is either distilled off or is aerated into an acid medium and the ammonia determined either by Nesslerization or by titration. Either of these expedients has the disadvantage of being cumbersome and time-consuming.

To overcome these difficulties Karr (4) recommended the direct Nesslerization of the blood filtrate after hydrolysis with urease, thus eliminating complicated steps in the procedure. This method, however, gives a cloudy solution when Nesslerized. Roe and Irish (5) have obviated this difficulty to a certain extent by adsorbing the interfering substances with calcium phosphate. In this procedure larger amounts of blood are necessary and an extra step is introduced in centrifugation and in transferring the material before Nesslerization. High urea contents will produce cloudy solutions in this method also.

In looking for a method that would be accurate, simple, and rapid we resorted to hydrolysis of urea by heat under pressure, thus eliminating the use of urease. Such a procedure has existed for some time and was advocated by Folin and Wu (6). It consists in heating the filtrate under pressure in an autoclave. Clark and Collip (7) have made use of this method; after hydrolyzing the urea they distil the ammonia in a special distilling apparatus into N HCl solution and titrate the excess acid with alkali.

This method is cumbersome and not easily applicable to routine clinical work. The hydrolysis of urea cannot be done in the ordinary bacteriological autoclave because a higher temperature is required than is obtainable with such an autoclave. The distillation is also an objectionable feature.

We have found that by hydrolyzing the urea under pressure in the presence of sulfuric acid direct Nesslerization is possible, thus eliminating the process of distillation or aeration. By using the modified Nessler solution of Koch and McMeekin (8) a perfectly clear solution is obtained. We substituted for the autoclave a special glass pressure tube devised by one of us (S. L. Leiboff) by means of which we were able to carry out our investigation to a successful conclusion.

*Apparatus for Hydrolysis of Urea (S. L. Leiboff).*[1]

The apparatus is illustrated in the accompanying drawing (Fig. 1). The pressure tube, *a*, is made of heavy Pyrex glass to withstand high pressure. The upper portion of the tube tapers off to a funnel shape and is ground on the inside at the tapered portion. The wide portion of the glass stopper inside the tube is ground in such a way that when the stopper is raised the two ground surfaces fit very snugly. When the temperature is raised to above 100° the pressure produced within the tube tightens the stopper very closely.

The heating is done in an oil bath, *d*. This bath consists of a metal container capable of holding six tubes, thus allowing simultaneous determinations. In the container is placed a removable metal rack, the center of which holds a 200° thermometer inclosed in a metal jacket to prevent breakage. To the upper part of the thermometer jacket is attached a circular disc, *b*, with six grooves into which the external ends of the stoppers slip, thus holding the pressure tubes suspended in the oil. The edges around the grooves are slightly turned upwards in order to prevent the tubes from falling off.

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[1] We are greatly indebted to Eimer and Amend, New York, for their cooperation in designing the apparatus.
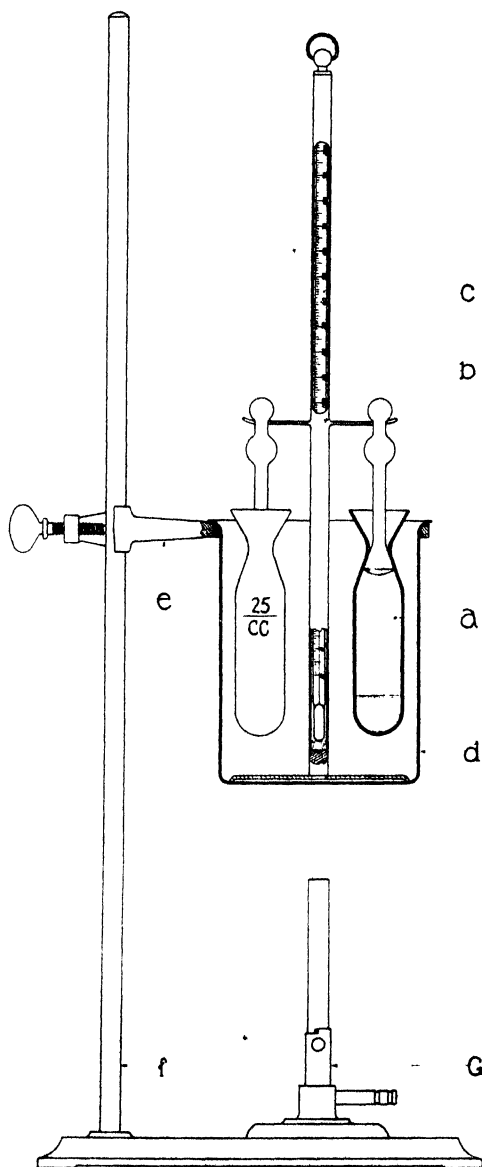
FIG. 1. Apparatus for hydrolysis of urea.

*Method for Determination of Urea Nitrogen.*

Suspend the pressure tube in the groove of the disc as shown in the diagram.    Insert the stopper half-way in the tube by raising the tube and introduce 5 cc. of Folin-Wu filtrate (1:10 dilution). Add 1 cc. of N sulfuric acid and wash down the stopper with 1 cc. of water.    The solutions are best introduced into the tube by holding the tip of the pipette close to the lower end of the stopper. Close the tube by holding the stopper in its place with one hand and pulling down the tube with the other hand, turning it slightly, thus making it fit snugly.    Place the rack holding the tube in the oil bath in such a way that the liquid in the tube is somewhat below the level of the oil.

Any oil of high boiling point will serve the purpose.    Nujol oil is satisfactory as it produces very little odor when heated.

The oil bath is heated with a Bunsen burner.    This was found preferable to an electric heater, as the temperature can thus be regulated very easily.    After a few preliminary trials one is able to adjust the size of the flame so that a constant temperature may be retained during the process of hydrolysis.    A variation of a few degrees does not matter.

The temperature of the oil is raised to 150° and is kept at that temperature for 10 minutes.    The tube is then removed from the oil bath and when cooled to room temperature add about 13 cc. of water and 3 cc. of modified Nessler solution (8).    Add water to the 25 cc. mark and invert the tube to mix the contents.    Compare in the colorimeter against a standard solution of ammonium sulfate.

*Standard Ammonium Sulfate.*

Dissolve 0.283 gm. of pure ammonium sulfate in 200 cc. of water in a liter volumetric flask and add N sulfuric acid to the liter mark. 5 cc. of this solution contain 0.3 mg. of nitrogen.

The standard for the test is prepared by introducing 5 cc. of the standard into a 100 cc. volumetric flask filled two-thirds with water.    While rotating the flask add 12 cc. of modified Nessler solution and fill up with water to the mark.

With the standard set at 20 mm. the following calculation is used.

$$\frac{S}{R} \times 15 = X$$

$S$ = reading of standard; $R$ = reading of unknown.

This method was checked against a large number of urea determinations performed by the urease aeration method, and gave very satisfactory results. The results were somewhat higher than

TABLE I.

*Comparison of New Method with That of Urease Aeration Method.*

| Blood sample No. | Urease method | New method. | Blood sample No. | Urease method. | New method. |
|---|---|---|---|---|---|
| | *mg.* | *mg.* | | *mg.* | *mg.* |
| 1 | 10.5 | 11.0 | 9 | 16.0 | 16.6 |
| 2 | 10.7 | 10.9 | 10 | 17.3 | 17.6 |
| 3 | 11.8 | 12.1 | 11 | 19.5 | 20.2 |
| 4 | 12.0 | 12.0 | 12 | 38.3 | 39.1 |
| 5 | 12.4 | 13.0 | 13 | 40.5 | 41.3 |
| 6 | 13.6 | 13.8 | 14 | 57.4 | 58.5 |
| 7 | 14.4 | 15.0 | 15 | 65.3 | 66.8 |
| 8 | 15.1 | 15.0 | | | |

TABLE II.

*Recovery of Urea.*

| Blood sample No. | Urea N added. | Total N present. | Amount of N found. |
|---|---|---|---|
| | *mg.* | *mg.* | *mg.* |
| 1 | 5 | 17.5 | 17.2 |
| 2 | 10 | 22.5 | 22.4 |
| 3 | 15 | 27.5 | 27.2 |
| 4 | 20 | 32.5 | 31.9 |
| 5 | 25 | 37.5 | 37.0 |
| 6 | 30 | 42.5 | 42.2 |
| 7 | 35 | 47.5 | 46.9 |

those obtained by the urease method. This we feel is due to greater accuracy in our method since no loss of ammonia is possible as with aeration or distillation. Folin and Wu (6) pointed out that the slightly higher figures obtained by hydrolysis of urea by heat are of no consequence for clinical work.

In Table I are given some of the figures obtained by the two methods.

*Recovery of Added Urea from Blood.*

A sample of blood containing 12.5 mg. of urea nitrogen per 100 cc. was divided into seven equal portions. To each portion was added a known increasing amount of urea. The proteins were then removed with tungstic acid in the usual way and the urea nitrogen determined by our method. Table II gives the amounts of urea recovered in terms of mg. of nitrogen per 100 cc. of blood.

The pressure tube used for the hydrolysis of urea is constructed to withstand a greater pressure than the one required in the method. It was heated in the oil to a temperature of 200° without bad effects.

When the temperature reaches close to 150° a very slight leak is produced due to the extreme pressure developed inside the tube. However, this is of no consequence and does in no way interfere with the quantitative recovery of urea nitrogen. Some tubes that were made during the process of experimentation and very similar to the present perfected tube were producing a great leak under high pressure. These leaky tubes were used in a number of determinations as controls and complete recovery of urea nitrogen was obtained with these tubes in spite of the leak.

## SUMMARY.

A method is described for the rapid and accurate determination of urea in blood. The urea solution is digested with acid in a special pressure tube for 10 minutes at 150°. By this means the urea is converted quantitatively into ammonia which is determined colorimetrically after the addition of Nessler's solution. No distillation or aeration of the ammonia is necessary.

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# NOTE ON A PREVIOUSLY UNDESCRIBED FORM OF TYROSINE CRYSTALS.

By JAMES C. ANDREWS.

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This note records a form of tyrosine crystal which appears not to have been previously described and which differs widely from the clusters of silky needles usually observed. It was first obtained during gradual neutralization of a very concentrated solution of tyrosine hydrochloride and it has been found easy to duplicate its formation by slight neutralization of a solution of the hydrochloride so concentrated as to start precipitation before neutrality to Congo red is reached. The crystal form consists of blunt prisms somewhat resembling crystals of aspartic acid, very different from the tyrosine needles usually observed. There is described below one of a number of such progressive neutralizations together with the properties of the various fractions of tyrosine obtained. In this case a nearly saturated solution of about 75 gm. of pure *l*-tyrosine in strong hydrochloric acid was neutralized with 25 cc. portions of 4.0 M NaOH. In order to demonstrate the purity of the different fractions Kjeldahl determinations were run on each, while the optical activity was determined to demonstrate that no question of optical isomerism is involved.

The first fraction obtained (Fig. 1) had an average nitrogen content[1] of 7.80 per cent and an optical activity of $[\alpha]_D^{25} = -9.5$. The mother liquor from this fraction had a bright blue reaction towards Congo red.

The second fraction (Fig. 2) had an average nitrogen content of 7.73 per cent and an optical activity of $[\alpha]_D^{25} = -9.7$. It will be observed that these crystals were still of the same prism form as

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[1] The nitrogen determinations were made by Miss Pauline D. Wyman.

the first fraction　The reaction of the mother liquor to Congo red was practically the same as above

The third fraction (Fig 3) had an average nitrogen content of 7 70 per cent and an optical activity of $[\alpha]_D^{25} = -10\,0$　The crys-



Fig 1　*l*-Tyrosine Fraction A × 333⅓　　Fig 2　*l*-Tyrosine Fraction B × 166



Fig 3　*l*-Tyrosine Fraction C × 166⅔　　Fig 4　*l*-Tyrosine Fraction D × 5

tals are obviously of a transition type between the prisms and the usual needles　The mother liquor was purple to Congo red

The fourth fraction (Fig 4) was of the form usually observed　It had an average nitrogen content of 7 63 per cent and an optical

activity of $[\alpha]_D^{25} = -10.0$.    The mother liquor from this fraction was nearly free from tyrosine and was nearly neutral to litmus.

The method of production of these fractions precludes the supposition that the crystals precipitated at low pH values could be the hydrochloride and the nitrogen values show definitely that only isoelectric tyrosine has been precipitated.    The theoretical nitrogen percentage of tyrosine is 7.73 per cent while that of the hydrochloride is 6.44 per cent.    The most likely source of contamination of tyrosine crystals is from cystine ($N = 11.67$ per cent) but all of these fractions gave negative sulfur tests.    The optical activity values are also practically constant and indicate nothing but $l$-tyrosine.    These values were obtained from 1 per cent solutions in 1.0 M HCl.

The melting points of these crystals constitute a further test of identity.    Samples of each of these four fractions were heated simultaneously in a bath and were found to melt (with decomposition) simultaneously at 289°.    Since in such cases, the rate of heating has considerable influence on the "melting point" this value may be considered in fair agreement with the previously recorded values of 272–314°.

To determine whether or not these crystals represent a fundamentally different crystal form, samples of Fractions A and B were submitted to Dr. George L. Keenan of the Microanalytical Laboratory of the United States Department of Agriculture for examination.    His report indicated that although the crystal form was unusual, the optical properties such as refractive indices and characters shown in polarized light were those of ordinary normal tyrosine.    It must be concluded that these crystals, which bear a superficial resemblance to aspartic acid, are merely a modification of the usual form of $l$-tyrosine.

# THE USE OF TUNGSTOMOLYBDIC ACID AS A PRECIPITANT FOR BLOOD PROTEINS.

By STANLEY R. BENEDICT AND ELEANOR B. NEWTON.

*(From the Department of Chemistry, Cornell University Medical College, New York City )*

In a recent paper (1), we suggested the use of molybdic acid as a precipitant for blood proteins. Aside from the advantage of making available an additional protein precipitant which may be of service under special conditions, the use of molybdic acid permits quantitative recovery of thioneine (ergothioneine) from blood. In the previous paper attention was called to the fact that some bloods showed a marked difference in sugar values when molybdic acid was used as a precipitant. This was found to be especially true with the latest Benedict copper method for blood sugar (2) and the question is being further studied.

In connection with work reported in another paper (3) related to the isolation of the non-sugar reducing substances from blood, we have had occasion to study further the question of protein precipitants. During the course of this work it has been found that while molybdic acid causes no loss of thioneine during precipitation of blood proteins, glutathione and perhaps other unknown blood constituents are precipitated to a considerable extent by the molybdic acid. During some work carried out by one of us which has not yet been completed on the preparation of colloidal molybdic and tungstic acids, it was noted that when a colloidal acid product was prepared from a mixture of sodium tungstate and sodium molybdate, this product had properties which could not be accounted for by regarding the substance as a simple physical mixture of molybdic and tungstic acids. This finding led us to try whether a mixture of sodium tungstate and sodium molybdate when used as a blood protein precipitant might not act differently from either one separately and yield filtrates which showed

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no loss of any of the known non-protein constituents. The results obtained appear to have fully justified this expectation.

The blood protein precipitant which we wish to recommend is prepared and used as follows: 10 gm. of pure, ammonia-free

TABLE I.

*Non-Protein Compounds in Mixed Oxalated Human Blood Samples after Tungstic Acid\* and Tungstomolybdic Acid Precipitation.*

All figures represent mg. per 100 cc. of blood.

| No of sample and acid used as precipitant. | Sugar. | | Uric acid. | | Thioneine. | Glutathione. | Non-protein N. | Creatinine |
|---|---|---|---|---|---|---|---|---|
| | Folin-Wu. | Benedict. | Direct. | Indirect. | | | | |
| 1  Tungstic. | 138 | 114 | 3.0 | 2.5 | 3.0 | 48 | | 1.7 |
| Tungstomolybdic. | 139 | 115 | 3.6 | 2.7 | 4.5 | 49 | 74 | 1.7 |
| 2. Tungstic. | 108 | 82 | 4.2 | 3.4 | 4.6 | 62 | 37 | 1.7 |
| Tungstomolybdic. | 111 | 79 | 4.9 | 3.2 | 9.3 | 68 | 36 | 1.7 |
| 3. Tungstic. | 126 | 86 | 4.0 | 3.1 | 5.1 | 89 | 49 | |
| Tungstomolybdic. | 130 | 87 | 4.8 | 3.3 | 10.1 | 90 | 49 | |
| 4. Tungstic. | 88 | 69 | 3.9 | 2.7 | 2.1 | 51 | 31 | 1.9 |
| Tungstomolybdic. | 87 | 71 | 5.0 | 3.2 | 5.0 | 54 | 31 | 1.9 |
| 5. Tungstic. | 115 | 84 | 3.9 | 3.3 | 3.5 | 88 | | |
| Tungstomolybdic. | 100 | 88 | 4.9 | 3.2 | 7.7 | 88 | | |
| 6. Tungstic. | 173 | 144 | 6.0 | 4.8 | 3.1 | 75 | | 2.3 |
| Tungstomolybdic. | 173 | 140 | 7.0 | 4.8 | 6.6 | 75 | | 2.2 |
| 7. Tungstic. | 162 | 121 | 4.5 | 3.7 | 2.9 | | 36 | |
| Tungstomolybdic. | 171 | 125 | 5.0 | 3.6 | 6.1 | | 38 | |
| 8. Tungstic. | 151 | 138 | 3.9 | 3.2 | 3.5 | 50 | | 2.2 |
| Tungstomolybdic. | 151 | 136 | 4.3 | 3.0 | 6.5 | 51 | | 2.2 |
| 9. Tungstic. | 170 | 135 | 4.5 | 3.3 | 3.9 | 83 | | |
| Tungstomolybdic. | 152 | 126 | 5.1 | 3.7 | 6.0 | 83 | | |
| 10. Tungstic. | 150 | 128 | 7.1 | 3.3 | 19.6 | 64 | | |
| Tungstomolybdic. | 150 | 122 | 7.6 | 3.3 | 24.4 | 72 | | |

\* The sodium tungstate employed in this work was first proved to be free from molybdate.

molybdic acid are treated in a flask with 50 cc. of n sodium hydroxide solution and the mixture boiled for 1 to 2 minutes. A practically clear solution should result. About 150 cc. of water are added and the cooled solution is mixed with a solution of 80 gm. of sodium tungstate dissolved in about 600 cc. of water. This

mixed solution is diluted to 1 liter. The acid employed during the precipitation is 0.62 N sulfuric acid, and is prepared by diluting 620 cc. of N acid to 1 liter. The precipitation of the blood proteins is carried out exactly as in the familiar tungstic acid precipitation. The blood is diluted with 7 volumes of water, 1 volume of the mixed tungstate-molybdate solution is added, followed by 1 volume of the 0.62 N sulfuric acid.

We have made numerous comparative determinations and have found that the new precipitating mixture does not cause precipitation or loss of any known non-protein constituents of the blood. Illustrative figures for human blood are given in Table I. It will be noted that non-protein nitrogen and creatinine figures agree almost exactly by the two procedures. From the increased amount of thioneine present it might be assumed that total nitrogen would tend to be detectably higher in the tungstomolybdic filtrate. The fact that no such difference in the figures is apparent may be partially accounted for by a more complete removal of protein in the case of tungstomolybdic filtrates. Most of our blood samples were heavily oxalated and as a general rule the tungstomolybdic filtrates showed distinctly less tendency to froth than did the tungstic acid filtrates, indicating the presence of less protein. We believe that in this fact may also lie a partial explanation of the great difference in sugar values noted in Sample 9 of Table I. The blood proteins exhibit some reducing action, and may have contributed to the very unusual results obtained with this sample of blood. We are studying further the question of the sugar values obtained where the two different protein precipitants are employed. The figures for glutathione agree closely by the two procedures, though these figures must be regarded as of *very questionable accuracy*. They depend on a comparison of the intensity of color yielded by the nitroprusside reaction after precipitation of the glutathione with silver and solution of this precipitate in cyanide. We are doubtful whether even a reasonably accurate method can be worked out on this basis, which is a modified form of the one adopted by Hunter and Eagles (4). It will be noted in the table that the figures for thioneine average very much higher where the tungstomolybdic acid has been employed than where tungstic acid alone is used. The tremendous differences between thioneine values following these two precipi-

tants lead one to question whether such figures for thioneine as those reported by Eagles and Vars (5), using tungstic acid precipitation, are of any special significance.

In connection with Table I it is further of interest to note that the uric acid figures by the direct method are appreciably higher where the tungstomolybdic precipitant is used. This is undoubtedly chiefly due to the presence of the increased amount of thioneine in these filtrates, but we have reason to believe that one or two other unknown compounds are also here involved. It will be noted that where the indirect method is employed figures agree excellently for the two methods of precipitation. We hope in the near future to report upon a new uric acid reagent which is so specific for uric acid as to permit its direct determination on the tungston.olybdic filtrates.

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# STUDIES ON THE NON-SUGAR REDUCING SUBSTANCES
## OF THE BLOOD AND URINE.

### I. GLUTATHIONE AND THIONEINE IN BLOOD.

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*(From the Department of Chemistry, Cornell University Medical College, New York City.)*

During a study of the precipitation of various substances from blood filtrates by means of an excess of silver salts in the presence of a small amount of sodium tungstate, it was found that practically all of the non-glucose reducing materials present in many blood filtrates were completely removed by this treatment. Thus we were led to attempt the isolation of one or more of the compounds responsible for the residual reduction yielded by blood. During the present isolation study we desired to avoid the presence of thioneine in so far as possible. Hence sheep blood was used since it contains relatively little thioneine (1 to 4 mg. per 100 cc.), and tungstic acid was employed as the protein precipitant because this reagent precipitates a large proportion of whatever thioneine may originally be present in the blood. We shall discuss the relationship of thioneine to the residual reduction of blood later in this paper.

After the initial precipitation of the sheep blood filtrate with excess of the Folin-Wu silver lactate solution, it was found that all of the non-glucose reducing material originally present in the blood was contained in the precipitate.[1] Various procedures were

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[1] It is interesting to note that the rest reduction of sheep blood after fermentation usually averages about 22 mg. per 100 cc., while following incubation overnight the residual reduction is much lower, figures of 12 to 14 mg. per 100 cc. being obtained. Experiments in which glutathione was added to sheep blood, which was then incubated, have indicated that a considerable proportion of glutathione disappears during incubation. We are studying further the points here involved.

used in connection with the problem of isolating the reducing
substance, each step being checked by the sugar reduction value
of the various fractions.    This work finally resulted in the isola-
tion of glutathione, the quantity obtained being considerably
more than double the amount isolated from sheep blood by Holden
(1) or by Hunter and Eagles (2).    The work also resulted in
showing for the first time that glutathione is the substance chiefly
responsible for the non-glucose reduction yielded by blood filtrates.
Somogyi (3) has suggested that glutathione might contribute to
the rest reduction of blood, though he had no evidence for this
assumption other than the fact that glutathione occurs in blood
as demonstrated by Holden (1) and by Hunter and Eagles (2).
Apparently no studies of the reducing action of glutathione toward
sugar reagents have hitherto  been reported.    We will report
upon this question later in this paper.

The procedure finally adopted for the isolation of glutathione
from sheep blood is simpler than any method hitherto reported
and the yield is higher, so that we feel it worth while to report
the procedure in detail.    Indeed we are of the opinion that sheep
blood is the best source of glutathione at present available, being
superior to yeast for this purpose.

The procedure for isolation of glutathione from sheep blood is
as follows: We have handled 6 to 8 liters of the fresh defibrinated
blood at one time.    The blood is diluted with water and precipi-
tated in 1:10 dilution according to the familiar Folin-Wu tungstic
acid precipitation method, and poured upon large filters.    The
clear filtrate is placed in tall cylinders and mixed with 0.1 its
volume of the Folin-Wu solution of silver lactate in lactic acid.
The mixture is protected from light and allowed to settle for
about 4 hours.    The supernatant fluid is then decanted and the
remaining thick suspension of silver salts is centrifuged.    The
supernatant fluid is discarded and the residue in the tubes is
washed once by stirring with water, followed by centrifugation.
The wash water is poured off and the precipitate stirred thoroughly
with 0.4 its volume of the Folin-Wu solution of 10 per cent sodium
chloride in 0.1 N hydrochloric acid.    This mixture is centrifuged
and the supernatant liquid discarded.    The residue in the centri-
fuge tubes is washed three or four times by stirring with water
followed by centrifugation.    The residue in the centrifuge tubes

is then mixed with boiling 0.5 N hydrochloric acid, the volume of acid used being equal to about 0.2 that of the original blood. The mixture is then boiled for not more than 5 minutes, filtered at once, and the filtrate placed in a refrigerator overnight. A slight precipitate usually forms during this time, which should be removed by filtration. The filtrate is treated with a few pieces of granulated zinc and a piece of platinum for about 20 minutes and again filtered. This treatment removes traces of tungsten and of silver. The filtrate is shaken with about 0.1 its weight of mercuric acetate. The abundant flocculent precipitate which forms is allowed to settle for an hour and the mixture is then centrifuged. The residue of mercury salts in the centrifuge tubes is washed twice by thorough stirring with 1.5 per cent mercuric chloride solution and once with alcohol. The residue is then suspended in a volume of water equal to about 0.16 of the volume of the original blood and this mixture treated with an excess of hydrogen sulfide. The resulting mercury sulfide is then removed by filtration and the excess hydrogen sulfide is removed by an air current. The solution thus obtained is precipitated with 10 per cent mercuric sulfate in 7 per cent sulfuric acid (Hopkins-Cole reagent). The mercury reagent should be added slowly with stirring until precipitation appears to be complete. The flocculent precipitate is centrifuged off after an hour and washed three times with water in the centrifuge tubes. The residue in the tubes is then suspended in water (0.1 volume of the original blood) and decomposed with an excess of hydrogen sulfide. The mercury sulfide and hydrogen sulfide are then removed as before. The solution is now freed from sulfuric acid by addition of the theoretical quantity of barium hydroxide solution, the quantity of barium necessary being determined by titration of a portion of the solution. After centrifuging off the precipitated barium sulfate, the neutral solution of glutathione is evaporated to dryness under reduced pressure, the temperature not being allowed to go over 60°. The crude glutathione which separates as a glaze on the walls of the flask is taken up in about 3 cc. of water and the solution centrifuged free from traces of barium sulfate. The clear solution is then poured drop by drop and with constant stirring into 30 to 40 cc. of absolute alcohol. The glutathione separates at once as an amorphous fluffy precipitate. The mixture should be allowed

to stand overnight in a refrigerator and then centrifuged and the glutathione washed twice with absolute alcohol and then with ether. The product may then be dried in an air bath to remove the remaining alcohol and ether. While this product is nearly pure, it is best to reprecipitate it with alcohol and ether from a concentrated aqueous solution.

The yield obtained by this method is at least 0.1 gm. of glutathione per liter of blood. In comparing this yield with those of Holden (1) and of Hunter and Eagles (2), it should be noted that while these other investigators extracted the original precipitate of blood proteins, our procedure involves an initial loss of about 25 per cent since we use only that portion of the blood filtrate obtained by filtration overnight.

The glutathione prepared by this method yielded 12.12 per cent of nitrogen against theoretical 11.29 per cent, and 11.21 per cent of sulfur against theoretical 12.9 per cent.

The high nitrogen and low sulfur content of our preparation is in general agreement with the findings reported by Hunter and Eagles (2). We were unable to obtain figures agreeing more closely with the theoretical by further purification (reprecipitation with mercury, copper hydrate precipitation). We are inclined to the view expressed by Hunter and Eagles (4) that the formula usually ascribed to glutathione is not correct, or we must believe that a certain impurity clings to the glutathione with great tenacity. This is of course not very improbable where, as in this case, the compound was not purified by recrystallization.

The glutathione which we prepared showed a reducing action upon the Folin-Wu copper reagent equivalent to almost exactly 0.2 its weight of glucose. A sample of glutathione, for which we are indebted to Dr. H. D. Dakin, showed an essentially identical reducing value toward the Folin-Wu reagent. Since glutathione apparently occurs in blood in amounts between 50 and 100 mg. per 100 cc., we are justified in assuming that this substance is responsible for a large proportion of the non-sugar reduction yielded by blood. We are not prepared at the present time to report upon the reducing value of glutathione in terms of glucose with other sugar reagents than the Folin-Wu solution. Our studies in this connection have so far yielded results which are so difficult to interpret that we are not prepared to report upon

them at present. Apparently both the reduced and oxidized forms of glutathione have very nearly identical reducing values with the Folin-Wu reagent. 50 mg. of glutathione added to 100 cc. of sheep blood and yielding 10 mg. as reducing sugar were almost exactly recovered by the increased reduction of the Folin-Wu reagent, but caused no increase in reducing power of the blood filtrate by the new Benedict solution. This is in accord with the findings reported by one of us (5) that in the presence of much glucose the non-sugar reducing substances of blood do not appreciably affect the Benedict copper solution.

In a previous paper (6) we stated that thioneine does not cause formation of visible cuprous oxide when heated with alkaline copper solutions. We have since found, however, that it does cause some reduction. When thioneine is heated in the Folin-Wu sugar method, though there is no visible precipitation of cuprous oxide, a final color results when the color reagent is added which indicates that thioneine has, like glutathione, a reducing power equal to about 0.2 its weight of glucose. In studying the behavior of thioneine with the Folin-Wu reagent we have noted further that thioneine causes some reduction *in the cold*, and that it is indeed capable of developing color in the phosphomolybdic color reagent without intermediate action upon copper. Nevertheless, the total reducing power of thioneine is so low that the quantity usually present in blood does not account for more than 1 to 2 mg. or occasionally as much as 4 to 5 mg. of the total rest reduction.

We are studying further the questions involved in the rest reduction of the blood and urine. Apparently human blood has at least one, or probably two reducing non-sugar compounds besides glutathione and thioneine.

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# OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL SIGNIFICANCE.

## V. THE COMPOSITION OF THE OXIDIZED COBALT COMPLEX OF CYSTEINE. A COLORIMETRIC METHOD FOR THE MICRO ANALYSIS OF COBALT.

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In a previous communication (1) a cobalt complex of cysteine has been described which arises from cysteine and a cobalt salt in weakly alkaline solution on exposure to the air. A gas analytical method showed that this complex contains cobalt atoms and cysteine molecules in the ratio approximately 1:3 and consumes, when prepared from its constituents, two-thirds of the amount of oxygen which would have been necessary to oxidize all its cysteine to cystine, or double as much oxygen as would have been necessary to oxidize the cobaltous state to the cobaltic. The ratio of cobalt to cysteine could, however, only approximately be determined by this method for reasons set forth in the above paper. As the accurate knowledge of this complex compound will be shown to be most important for a theory of the catalytic oxidation of cysteine to be developed later, another method was used to ascertain the constitution of this complex. The attempts to precipitate this easily soluble, brown compound and to analyze either this complex acid itself or one of its salts in pure condition has failed so far. The precipitate, *e.g.* as obtained in a great excess of alcohol, yielded an amorphous compound which, redissolved in water, showed a somewhat more green shade, contained cysteine and cobalt in a ratio smaller (1:2.2 to 1:2.5) than expected from the gas analytical data, and was obviously different from the original complex in the solution. Possibly it is not even a single well characterized chemical substance. For the time being, the

preparation of this complex in pure condition has been abandoned, and the description of the attempts of its preparation in pure and dry condition may be omitted.   Instead another method was used
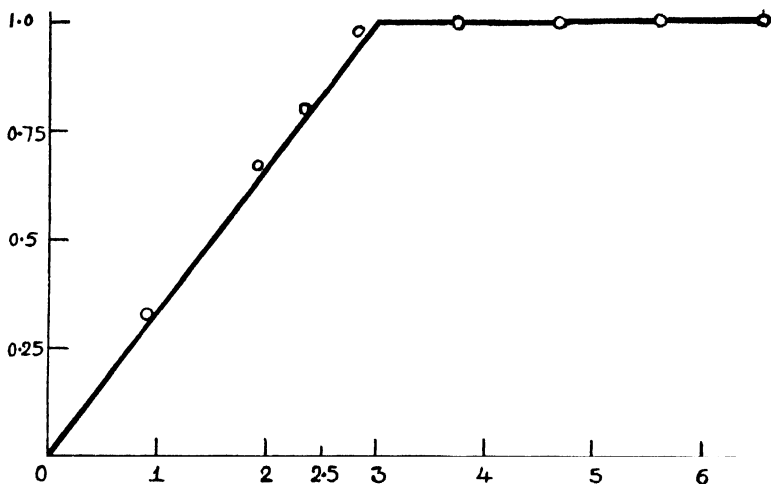


Fig. 1. 1 cc. of 0.01 M solution of $CoSO_4$ is mixed with varied amounts in cc., indicated on the abscissa, of 0.01 M solution of cysteine hydrochloride.   22 cc. of phosphate buffer (M/15 solutions according to Sörensen), pH = 7.4, are added to each, and enough 0.01 M HCl to make up a volume of 30 cc.   The ordinates show the color intensity, the tube with the greatest quantity of cysteine being taken as the color standard.   The readings were made 2 hours after the preparation of the solutions.   The drawn out curve is the one calculated for the complex with 1 atom of cobalt to 3 molecules of cysteine.

The preparation of cysteine hydrochloride contained 93 per cent of the expected amount of cysteine, both according to the oxygen consumption with iron as catalyst and to iodine titration according to Warburg.   The latter was performed by dissolving a weighed amount of cysteine hydrochloride (around 30 mg.) in 0.5 cc. of water, adding 5 cc. of 95 per cent alcohol, and titrating with 0.05 N alcoholic iodine solution without starch. The amount of cysteine in the diagram is calculated according to this titer of the cysteine preparation.

to ascertain the ratio of cobalt to cysteine in the complex as it arises in the solution.   This method is based on the following principle.   When a constant amount of a cobalt salt, in the presence of a phosphate buffer suitable for the formation of the wanted

complex, is mixed with increasing amounts of cysteine and exposed to the air, the depth of color of the solution due to the formation of the complex will increase with increasing amount of cysteine. If it be true that under these conditions only one kind of complex is formed, there will be no difference in shade with varied amounts of cysteine, but only in color intensity. When that amount of cysteine is reached which can just combine with the amount of cobalt used, the color intensity will have a maximum which will not be exceeded by adding more cysteine, provided the affinity of the complex formation is high and the formation of the complex is practically complete. In fact, it could be shown that such a maximum is reached when the ratio of cobalt to cysteine is exactly 1:3. (See Fig 1.) With a ratio 1:2, the color intensity is two-thirds of the maximum, with a ratio 1:1 it is one-third of the maximum. Hence it may be taken as certain that the oxidized cobalt complex contains Co and cysteine in the ratio exactly 1:3.

The color of this complex is very stable as has been said. On observing these solutions, kept in the dark over a period of days, it can be seen by colorimetric comparison of freshly prepared and aged solutions that the color slowly decreases, especially in those tubes in which the ratio of cysteine to cobalt is smaller or equal to 3:1. It takes more than a week to reduce the color intensity to half its original degree; in those tubes containing an excess of cysteine the fading is even still slower. Two explanations for this phenomenon are imaginable. The first assumption is that the cysteine combined in the cobalt complex is very gradually and irreversibly destroyed. The second assumption may be that contaminating traces of iron salts behave as catalysts. The cysteine, though strongly combined with cobalt, may still not be strongly enough combined to escape entirely the competitive affinity of iron, which must lead to catalytic oxidation of the cysteine, in the presence of air. In parallel experiments with cobalt and cysteine in phosphate buffer of pH 7.5, one as iron-free as possible, the other with addition of ferrous sulfate to an amount of one-tenth of the cobalt, the rate of fading of the brown color, observed over a week, was the same. It seems, therefore, that the affinity of cysteine for cobalt is much greater than for iron, and that the fading is due to a very gradual destruction of the cysteine within the cobalt complex. In any case, the brown complex is

stable for many hours in solution, and we may assume it to represent, if not an absolutely stable, yet a well characterized product of the reaction between cysteine and cobalt in presence of oxygen.

The oxidation which is necessary to produce this complex from a cobaltous salt and cysteine can be brought about either by molecular oxygen or by oxygen-free oxidants. This fact makes it very likely that this oxidation is not a combination with oxygen, but a dehydrogenation. In the case of some cobalt complexes, son.etimes an addition of oxygen takes place leading to what is called the oxycobaltiacs and the anhydrooxycobaltiacs (2). The oxygen in these compounds is, however, rather loosely combined and easily detached. This is not the case with the oxidized cobalt-cysteine complex which is a very stable compound and could not be reduced by any means tried. This may be considered as sufficient proof for the assumption that this complex is not an oxygen-addition compound comparable with an oxycobaltiac, but a dehydrogenation product of a cobaltous compound. It has been shown in the previous communication (1) that when cobaltous cysteine, which is practically colorless in high dilution, is exposed to air, the color arising is in the beginning an olive-green shade and very soon becomes brown. The olive-colored compound is so transient that there is no hope to separate or analyze it. It is quite possible that the olive-colored complex is an addition product of the cobaltous complex with molecular oxygen, corresponding even in shade to the color of the carbon monoxide complex of cobaltous cysteine described by Cremer (3) and that this oxygen compound is a stage preliminary to the stable oxidized cobalt complex, which in its turn, certainly does not contain loosely combined oxygen.

It can furthermore be taken for a certainty that the complex does not contain any anion of the buffer used, phosphate or borate. For the formation of this complex takes place in absence of any buffer just the same provided the solution of any cobaltous salt (sulfate or nitrate) and of cysteine hydrochloride is made slightly alkaline with a carefully adjusted amount of NaOH. The very small amount of HCl, contained in cysteine hydrochloride, or of $SO_4$ contained in the cobalt sulfate will certainly not enter into the complex formation in competition with the much stronger complex former represented by the sulfhydryl compound.

These facts suggest a formulation for the complex compound. The reaction, leading to its formation, can be written:

$$CoX_2 + 3\ HSCH_2 \cdot NH_2CH \cdot COOH + \tfrac{1}{2}\ O_2 \rightarrow Co(SCH_2 \cdot NH_2CH \cdot COO)_3H_3 + 2XH + H_2O$$
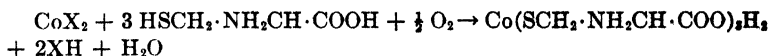
where X is any ordinary monovalent acid rest or OH. All six coordination places of cobalt are occupied by 3 molecules of cysteine. The interpretation of this formula, attributed hereby to the oxidized cobalt complex, with respect to its structure, will be discussed in a subsequent communication.

### Colorimetric Method of Micro Analysis for Cobalt.

It may be added that the formation of this complex can be used for a very sensitive and accurate method for the micro analysis of cobalt, which will be desirable even in biochemistry. Though several colorimetric methods for the analysis of cobalt have been described (4, 5), this one seems to be of great advantage. The method consists in the following procedure. The cobalt compound to be analyzed, in such an amount as to contain no more than 5 mg. of cobalt, is heated in a platinum crucible, containing about 1.5 cc. of concentrated $H_2SO_4$. The platinum crucible is placed in a larger nickel crucible and heated to dryness. After cooling, 25 cc. of phosphate buffer (Sörensen) pH 7.5 and cysteine hydrochloride crystals in excess (i.e. about 10 mg.) are added and the oxidation of the cobalt complex is accomplished by gently shaking this solution in a beaker so as to expose it to the air. The oxidation is completed in a minute or so, and the brown color is stable over many hours and longer. It can be compared in the colorimeter with a solution freshly prepared in the same way from a known amount of cobalt sulfate, which need not be heated with $H_2SO_4$. The cobalt sulfate used as standard should be practically free from nickel and iron, and recrystallized from a good preparation by allowing an aqueous solution, saturated at room temperature, to evaporate at room temperature to dryness. The crystals thus obtained are $CoSO_4 + 7\ H_2O$ and are very stable. The amount of cobalt used in the test should be chosen so as to match in color approximately that of the solution to be analyzed, according to the general principle of colorimetry. The color is

approximately the same as in Nesslerization of $NH_3$ and is very suitable for colorimetric readings.   Analyses of known amounts of cobalt in the range of a fraction of 1 mg. up to 5 mg. were correct within at least ± 5 per cent.   If necessary, the volume of the solutions can be made much smaller, and the accuracy of the quantitative determination remains the same in the analysis of a total amount of $\frac{1}{40}$ mg. of Co.   The presence of iron does not interfere with this method, even when there is a considerable excess of iron.   The violet-colored iron complex of cysteine is so transient in the presence of air, especially in such a pH range of phosphate buffer as used in the method, that it fades out gradually and does not interfere with the color of the cobalt complex.   The same is true for Cu and Mn.   The presence of nickel does not interfere either, even when nickel is present in equal amount as cobalt, because the reddish color of the nickel complex is extremely weak in comparison with the one of the cobalt complex.   In a ratio Co:Ni = 1:2 a very slight change of color can just be detected.   The presence of such a large excess of nickel which may disturb the determination of cobalt is betrayed by a change of the shade of color, but probably for biological purposes this case may not be important, as Bertrand and Macheboeuf in general find cobalt in a slight excess over nickel in the ashes of tissues.   The fact that in general the removal of nickel is unnecessary imparts a considerable advantage to this method compared with the method applied by Bertrand and Macheboeuf.

### SUMMARY.

1. It is ascertained by a colorimetric method that the oxidized cobalt complex of cysteine contains cobalt and cysteine in the ratio of 1:3.   This fact in combination with the amount of oxygen necessary to obtain this complex from its constituents suggests as formula for the oxidized cobalt complex of cysteine:

$$Co(SCH_2 \cdot NH_2CH \cdot COO)_3H_2$$

2. A colorimetric method for the micro analysis of cobalt is described which is based on the formation of this complex.

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# THE EFFECT OF AVITAMINOSIS ON HEMATOPOIETIC FUNCTION.

## I. VITAMIN A DEFICIENCY.*

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During the past few years medical science has made rapid advances in the therapeutics of pernicious anemia by dietotherapy. Stimulated by the researches of Whipple and Robscheit-Robbins (1) on the influence of the diet on blood regeneration in severe anemia in the dog, Minot and Murphy (2) have achieved marked success with liver diets in most accentuated cases of pernicious anemia. Cohn and coworkers (3) have concentrated the active principle in a water-soluble fraction of the liver. Koessler and Maurer (4) have secured equal success in the cure of this anemia with a high calorific diet, rich in vitamins, and particularly emphasize the need of vitamin A. As a matter of fact, Koessler, Maurer, and Loughlin (5) claim that blood regeneration cannot take place in pernicious anemia without the presence of vitamin A. They state that the blood changes in the gastrointestinal tract may be due to vitamin A underfeeding for a long period of years; the nervous symptoms may be due to an absence or deficiency of vitamin B, and the tendency to hemorrhages found in severe anemias may be due to the partial or complete lack of vitamin C. Later Hanke and Koessler (6) found little support for the theory of Koessler and coworkers in so far as the relation of the antiscorbutic vitamin to anemia is concerned. Out of four guinea pigs that developed a typical scurvy picture on an autoclaved soy bean ration only one developed anemia.

Since liver is a storage organ for vitamin B (7), and since liver is a specific factor for pernicious anemia, the relation of vitamin B

* Research Paper No. 123, Journal Series, University of Arkansas.

deficiency to such anemia readily suggests itself.   In 1922, Happ (8), however, reported no such relationship.   His conclusions were based on results with only two rats.   Happ further concluded that "diets deficient in vitamin A do not produce anemia in the rat; diets low in an organic substance contained especially in cod liver oil with a low calcium but high phosphorus content, which produce rickets-like changes in the rat, may also produce an anemia, provided the animal is kept for a long period on the diet; a diet low in the organic substance contained in cod liver oil and low in phosphorus with a normal calcium content, a diet that produces severe rickets with great uniformity, does not produce anemia."   If by the *organic substance* Happ refers to what is now recognized as the antirachitic dietary essential, vitamin D, then it is quite apparent that he was dealing with complicated avitaminosis, since no adequate provision was made for vitamin A.

Recently Cartland and Koch (9) reported: "Rats fed for weeks on diets deficient in Vitamins A, B, or E do not become anemic. Furthermore, we have found that such rats when subjected to severe experimental anemia by bleeding, can regenerate their blood completely every eight to fifteen days.   This rate is no lower than that observed upon similar diets complete in vitamins.   We conclude that vitamins A and E and probably vitamin B, are not specifically essential for the hemoglobin forming process in the rat. The experimental evidence with reference to the value of vitamin B in experimental anemia in rats is not conclusive."   For their vitamin B investigation only three animals were studied.   Neither Koessler and coworkers (5) or Cartland and Koch (9) in their vitamin A studies in relation to anemia have made ample provision for vitamin D.

We became interested in this problem in connection with our investigations of biochemical changes in the blood of nursing young of the albino rat suffering from vitamin deficiencies (10), which researches were stimulated by the suggestion made in 1925 by Hart and coworkers (11) that the infant mortality encountered on milk powder diets may be due to a deficiency of vitamin E which may function in the synthesis of hemoglobin.

In this paper, the first of a series on the effect of avitaminosis on hematopoietic function, we are reporting on the influence of uncomplicated vitamin A deficiency on the concentration of hemoglobin and erythrocytes in the blood of the albino rat.

Eleven animals whose mothers (third generation) subsisted on stock Diet 2 (12) were transferred at weaning to Ration 1034 of the following composition: casein (hot alcohol-extracted) 20; Northwestern dehydrated yeast 10; Salts 185 (13) 4; dextrin 66. The ration was irradiated for 30 minutes with a quartz mercury vapor lamp at a distance of 24 inches to insure an adequacy of vitamin D. This ration was now satisfactory in every respect with the exception of vitamin A. The animals were weighed daily and a record was kept of daily food consumption. The hemoglobin determinations and erythrocyte counts were made according to the technique described in previous publications (10, 12). The animals were at first bled every 12 days, subsequently weekly, and as the avitaminosis became more marked, several times a week. For a criterion of the severity of the vitamin A deficiency we have used the ophthalmic lesions. In order to obtain some information on blood concentration, we sacrificed the animals in different stages of the avitaminosis and secured enough blood from the carotid artery for the determination of total blood solids.

A careful examination of our food consumption records disclosed the fact that during the accentuated condition of vitamin A deficiency accompanied by ophthalmia marked inanition sets in, so that for several days the animal may eat nothing or only 1 to 2 gm. daily. In some animals that inanition period is followed by a resumption of food intake which may last from 4 to 8 days to be followed again by a loss of appetite. In other animals the anorexia is permanent. For this reason we are showing the results of three typical cases graphically, accompanying the data on hematopoietic function with graphs indicating the daily food consumption. The results on the rest of the eight animals are shown in Table II.

After transferring the young animals from their maternal diet to synthetic Ration 1034, composed of purified food substances, all the animals required 1 to 2 days to adjust themselves to the new diet before they began eating any appreciable amounts of it. Following that period they began to partake liberally of that ration, consuming 6 to 14 gm. daily until vitamin depletion became apparent. It is of interest to note that the three individuals that manifested no signs of ophthalmia also showed relatively little disturbance in food consumption. The temporary resumption of

food intake during the advanced stages of the avitaminosis may be due to the releasing of vitamin A by catabolism of tissues which function as storage organs.   That vitamin A, as well as vitamin B, exerts a specific influence on food intake has been shown by one of us (B. S.) in a recent publication (14).   We now have additional and more exact evidence to corroborate this fact.   Such findings are in agreement with those of Koessler and coworkers (5).

When animals are bled once weekly there is no object in accumulating daily food consumption records, but when biochemical changes are studied in the blood at more frequent intervals, par-

TABLE I.

*Effect of Age and Body Weight on Concentration of Hemoglobin and Erythrocytes in Control Animals (Ration 1145).*

| Age. | No of animals. | Average weight of each animal. | Hb | Erythrocytes | Total blood solids. |
|---|---|---|---|---|---|
| *days* | | *gm* | *gm  per 100 cc* | *millions per c mm* | *per cent* |
| 32 | 22 | 63 5 | 14 00 | 6 25 | 15 5 |
| 45 | 24 | 85 0 | 14 00 | 7 20 | |
| 50 | 18 | 110 0 | 14 50 | 7 55 | |
| 62 | 18 | 140 0 | 14 75 | 8 10 | |
| 66 | 18 | 150 0 | 15 00 | 8 00 | |
| 70 | 18 | 160 0 | 15 50 | 7 75 | |
| 85 | 12 | 185 0 | 15 00 | 8 25 | 17 0 |
| 114 | 30 | 214 0 | 14 90 | 9 08 | |

ticularly during the advanced stages of avitaminosis, we are convinced that it is impossible to interpret the blood chemistry without at the same time knowing the plane of nutrition.   For instance, it makes a great deal of difference whether, after making a blood sugar determination or a hemoglobin determination, an animal was consuming, the previous 1 to 2 days, nothing to 2 gm. daily, or 6 to 8 gm. daily; in other words, the investigator must be able to correlate the blood picture with optimum nutrition, inanition, or starvation.   With such facts on hand, we can now attempt to interpret the results on hematopoietic function of the eleven animals we subjected to vitamin A deficiency for 36 to 46 days.

It is quite apparent from Table I that the control animals that received Ration 1145 (which is a duplicate of Ration 1034, but
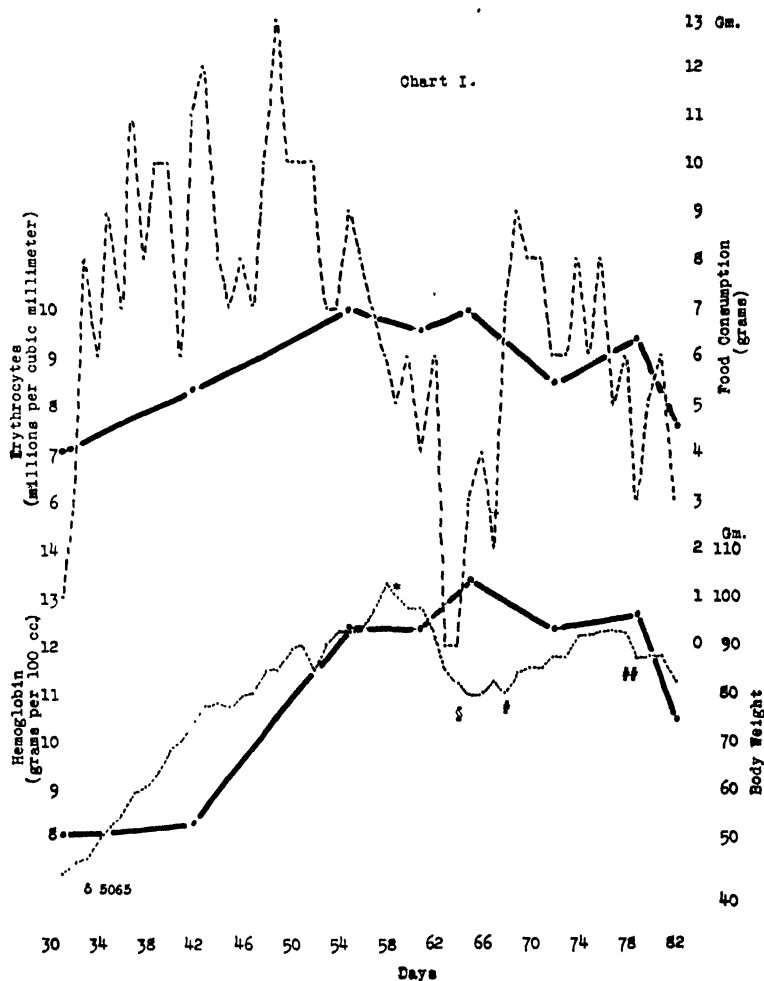
[CHART I. The effect of vitamin A deficiency on hematopoietic function of ♂ 5065. The upper curve in heavy lines represents erythrocytes expressed in millions per cmm. of blood. The lower curve in heavy lines represents hemoglobin expressed in gm. per 100 cc. of blood. The upper curve in dotted lines represents daily food consumption in gm. The lower curve in dotted lines represents body weight in gm.

* Indicates incipient ophthalmia in one eye; § marked ophthalmia in one eye; ⚥ severe ophthalmia in one eye; ⚥ ⚥ severe ophthalmia in both eyes.

contains in addition 5 per cent of butter fat) show, for ages ranging
from 32 to 85 days and corresponding weights of 63 to 185 gm.,
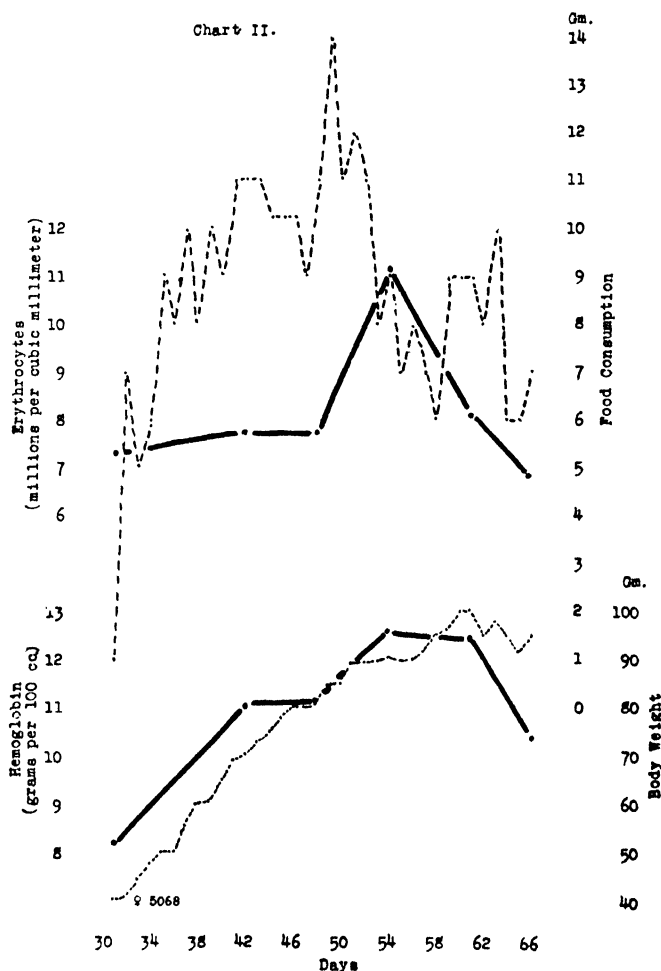


Chart II.

CHART II.   The effect of vitamin A deficiency on hematopoietic function
of ♀ 5068.   The upper curve in heavy lines represents erythrocytes ex-
pressed in millions per cmm. of blood.   The lower curve in heavy lines re-
presents hemoglobin expressed in gm. per 100 cc. of blood.   The upper curve
in dotted lines represents food consumption in gm.   The lower curve in
dotted lines represents body weight in gm.
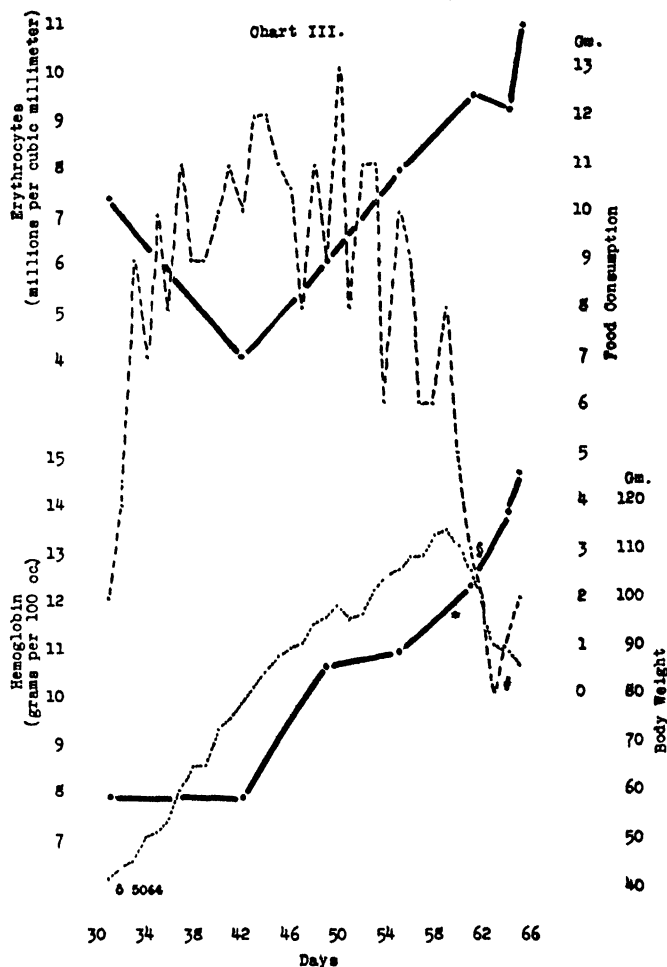
CHART III. The effect of vitamin A deficiency on hematopoietic function of ♂ 5064.    The upper curve in heavy lines represents erythrocytes expressed in millions per cmm. of blood.    The lower curve in heavy lines represents hemoglobin expressed in gm. per 100 cc. of blood.    The upper curve in dotted lines represents food consumption in gm.    The lower curve in dotted lines represents body weight in gm.

* Indicates incipient ophthalmia in one eye; § marked ophthalmia in one eye; # severe ophthalmia in one eye.

TABLE II.

*Effect of Vitamin A Deficiency on Hematopoietic Function of Growing Animals.*

| Animal No. | Age. | Weight. | Condition of animal. | Hb | Erythro-cytes. | Total blood solids. |
|---|---|---|---|---|---|---|
| | *days* | *gm.* | | *gm. per 100 cc.* | *millions per cmm.* | *per cent* |
| ♂ 5058 | 30 | 44 | | 8 18 | 5.64 | |
| | 42 | 71 | | 9.53 | 7.64 | |
| | 49 | 87 | | 11.07 | 8.84 | |
| | 55 | 97 | | 12.30 | 10.16 | |
| | 61 | 101 | * | 10.33 | 9.16 | |
| | 65 | 86 | † | 14.58 | 9.00 | |
| | 72 | 89 | † | 12.61 | 8.28 | |
| | 76 | 89 | *‡ | 15.07 | 8.48 | 22.6 |
| ♂ 5060 | 30 | 38 | | 8 61 | 5.64 | |
| | 42 | 63 | | 9.72 | 8.36 | |
| | 49 | 75 | | 11 68 | 8.76 | |
| | 55 | 89 | | 12 61 | 8.88 | |
| | 61 | 82 | * | 12 98 | 8.48 | |
| | 65 | 77 | ‡ | 16 66 | 9.32 | |
| | 66 | 71 | ‡ | 15 07 | 7.32 | 20.0 |
| ♀ 5061 | 30 | 44 | | 7.50 | 6.68 | |
| | 42 | 71 | | 10.15 | 7.08 | |
| | 49 | 75 | | 11.68 | 8.76 | |
| | 55 | 90 | | 12.98 | 9.12 | |
| | 61 | 97 | * | 12.98 | 10.00 | |
| | 65 | 87 | † | 13 34 | 12.64 | |
| | 74 | 81 | § | 14 58 | 8.76 | 20.9 |
| ♂ 5062 | 30 | 39 | | 8 30 | 6.60 | |
| | 42 | 73 | | 9.72 | 7.00 | |
| | 49 | 84 | | 11.38 | 7.72 | |
| | 55 | 96 | | 12 61 | 8.08 | |
| | 61 | 93 | * | 12 61 | 10.04 | |
| | 65 | 86 | ‡ | 14.15 | 10.08 | |
| | 66 | 83 | ‡ | 13.34 | 8.04 | 20.7 |

* Incipient ophthalmia in one eye.
† Marked ophthalmia in one eye.
‡ Severe ophthalmia in one eye.
§ Severe ophthalmia in both eyes.
Two different signs indicate two different conditions in the two eyes.

TABLE II—*Concluded*.

| Animal No. | Age. | Weight. | Condition of animal. | Hb | Erythrocytes. | Total blood solids. |
|---|---|---|---|---|---|---|
| | *days* | *gm.* | | *gm. per 100 cc.* | *millions per cmm.* | *per cent* |
| ♂ 5059 | 30 | 42 | | 7 87 | 4.64 | |
| | 42 | 68 | | 10.15 | 7.52 | |
| | 49 | 81 | | 10.82 | 7.68 | |
| | 55 | 90 | | 11 93 | 8 00 | |
| | 61 | 85 | * | 13.71 | 11.92 | |
| | 65 | 85 | † | 15 56 | 10.80 | |
| | 72 | 88 | †‡ | 12.30 | 12.20 | |
| | 74 | 83 | †‡ | 12.98 | 7.98 | 20.6 |
| ♀ 5066 | 31 | 36 | | 8.80 | 6.64 | |
| | 42 | 58 | | 8.06 | 8.24 | |
| | 55 | 73 | | 9.90 | 6.84 | |
| | 61 | 84 | | 15.07 | 7.64 | |
| | 65 | 86 | | 8.61 | 10.40 | |
| | 67 | 89 | | 10.15 | 6.76 | 19.7 |
| ♀ 5067 | 31 | 40 | | 9.90 | 8.52 | |
| | 42 | 56 | | 10.56 | 8.76 | |
| | 55 | 70 | | 9.53 | 8.52 | |
| | 61 | 82 | | 11.07 | 10.00 | |
| | 65 | 77 | | 10.56 | 11.48 | |
| | 67 | 76 | | 11.07 | 9.72 | 20.3 |
| ♀ 5063 | 30 | 40 | | 8.06 | 6.88 | |
| | 42 | 59 | | 8.48 | 6 84 | |
| | 55 | 74 | | 10.56 | 7.60 | |
| | 61 | 87 | * | 12.30 | 9.44 | |
| | 65 | 85 | † | 12.30 | 9.64 | |
| | 66 | 83 | † | 11 93 | 8.28 | 16.4 |

hemoglobin concentrations of 14 to 15 gm. per 100 cc. of blood, and erythrocyte counts of 6.25 to 8.25 millions per cmm. of blood. None of our control animals showed the degree of fluctuations in the hemoglobin or erythrocyte concentration as that encountered among the pathological animals.

*Chart I, ♂ 5065.*—From the period of the onset of ophthalmia there is a definite decrease in concentration of both hemoglobin and red blood corpuscles. The high peak in hemoglobin reached on the 65th day may be attributed to anhydremia produced by

inanition.   The per cent of total blood solids on the 82nd day, when the animal was sacrificed, was 20.4.   Compared with control animals, both from the standpoint of weight and age, vitamin A deficiency produced anemia and concentration of blood.

*Chart II*, ♀ *5068.*—This animal represents a typical case of progressive vitamin A deficiency preceding the development of eye lesions.   The hematopoietic disturbance produced becomes more evident when not complicated by marked reduction of food consumption.   Animals ♀ 5066 and ♀ 5067 showed similar results.   ♀ 5068 showed a concentration of total blood solids of 17.8 per cent which is within the range of normality.

*Chart III*, ♂ *5064.*—This animal shows a typical case of concentration of blood, or anhydremia, produced by a progressive marked inanition, as evidenced by the pronounced rise in the concentration of hemoglobin and erythrocytes.   On the day the animal was sacrificed the red blood corpuscles reached a count of 10.84 millions per cmm. which for an animal of that weight, 85 gm., should be 7.20 millions per cmm., and for an animal of that age (65 days) but weighing 150 gm. should be 8.00 millions per cmm.   The total blood solids on the 65th day when the animal was sacrificed was 22.3 per cent, or an increase over the normal of 31 per cent.

The results obtained on the rest of the eight animals shown in Table II are similar when interpreted in conjunction with the food intake.   There is a hematopoietic disturbance characterized by changes in concentration of either hemoglobin or erythrocytes or both.   The concentration of these blood constituents is influenced by the plane of nutrition, and in the early stages of vitamin A depletion preceding the onset of ophthalmia there is a suggestion of an anemia when the avitaminosis is not complicated by inanition.   We realize, however, that we do not have a sufficient number of clean-cut cases to establish that point, and are, therefore, continuing with this phase of the investigation.   There certainly is no relation between vitamin A deficiency and pernicious anemia, as claimed by Koessler, Maurer, and Loughlin (5).

### SUMMARY.

After vitamin A deficiency has progressed to the ophthalmic stage inanition complicates the blood picture, so that the high

figures of hemoglobin and erythrocytes may be an expression of anhydremia, indicated·by the concentration of total blood solids of the pathological animals as compared with normal animals of the same age and weight. In the preophthalmic stage there is a suggestion of an anemia characterized by reduction in either hemoglobin or erythrocytes. The results, however, are not conclusive and this phase of the problem is being studied further.

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# THE EFFECT OF AVITAMINOSIS ON HEMATOPOIETIC FUNCTION.

## II. VITAMIN B DEFICIENCY.*

By BARNETT SURE, M. C. KIK, and DOROTHY J. WALKER.

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ln recent communications we have demonstrated that nursing young of the albino rat suffering from a deficiency of the vitamin B complex (1) or from uncomplicated vitamin B deficiency (2) develop anhydremia associated with disturbance in hematopoietic function. The anemia encountered among such nurslings may have been produced by frequent bleedings, since it was necessary to remove at one time as much as 26 per cent of the total blood of the animal, in order to secure enough blood for determinations of the various blood constituents. Such young, however, regenerated their blood readily after vitamin B therapy.

Since we have no available method for the measurement of the amount of milk secreted by the rat, it is impossible to correlate the biochemical changes in the blood with the food intake of the nursing baby rat. All the information we have access to is the food consumption of the lactating mother. The mother may ingest an adequate amount of food and, because of vitamin deficiency, may produce an insufficient amount of milk or milk of poor quality for optimum welfare of the young, so that the exact plane of nutrition of the nurslings is an unknown factor. We, therefore, believe that biochemical changes in the blood of nursing rats suffering from avitaminosis should be followed up with similar studies on growing and adult animals on which correlations can be made between the food intake and the blood chemistry during various stages of avitaminosis. In this study we are dealing with such animals.

This investigation may be divided into two parts as follows:

*Part I.*—The effect of a deficiency of the vitamin B complex (vitamins B and G[1]) on hematopoietic function.    For this study we have employed two groups of animals.    In the first group we have used eighteen rats during the postlactation period, sixteen of which have failed to rear their young because of an insufficiency of the vitamin B complex for milk secretion.    Most of these mothers have either maintained or gained 2 to 5 per cent in body weight.    The two mothers that successfully reared their litters maintained their weight during lactation.    These eighteen animals were placed on Ration 1009 (3), deficient in the vitamin B complex, and after a week or two on this diet, ten females showed pronounced anemia as evidenced by the reduction of the concentration of hemoglobin and erythrocytes.    The hemoglobin was reduced to as low as 6.5 gm. per 100 cc., and the erythrocytes below 6 millions per cmm.    This anemia, however, was only temporary and lasted only from 4 to 10 days.    As the avitaminosis progressed, accompanied by loss of body weight, the concentration of both hemoglobin and red blood corpuscles increased, and in some cases, after a loss of as much as 40 per cent of body weight, the concentration of hemoglobin reached as high a figure as 19.4 gm. per 100 cc., and the erythrocytes over 11 millions per cmm.    In a recent communication we have shown (4) that on an adequate cereal ration (our standard dietary régime used for our breeding colony since 1921) an adult female rat shows a concentration of hemoglobin of 13.5 to 15 gm. per 100 cc., and an erythrocyte count of 8.5 to 9.5 millions per cmm.    The pathological group, therefore, indicated anhydremia.    In order to eliminate the influence the suboptimum diet for lactation might have on animals that later are subjected to vitamin depletion, and in order to follow the progress of the avitaminosis with measurements of blood concentration, we have selected another group of twenty-one females that successfully reared and weaned their litters, and for the first 3 to 6 weeks were allowed Ration 1145, containing 10 per cent Northwestern dehydrated yeast, which furnished an

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[1] We have now adopted the letter "G" to designate the stable, antipellagric factor, according to the recent decision of the committee on nomenclature of the American Society of Biological Chemists (*Science*, **69**, **276** (1929)).
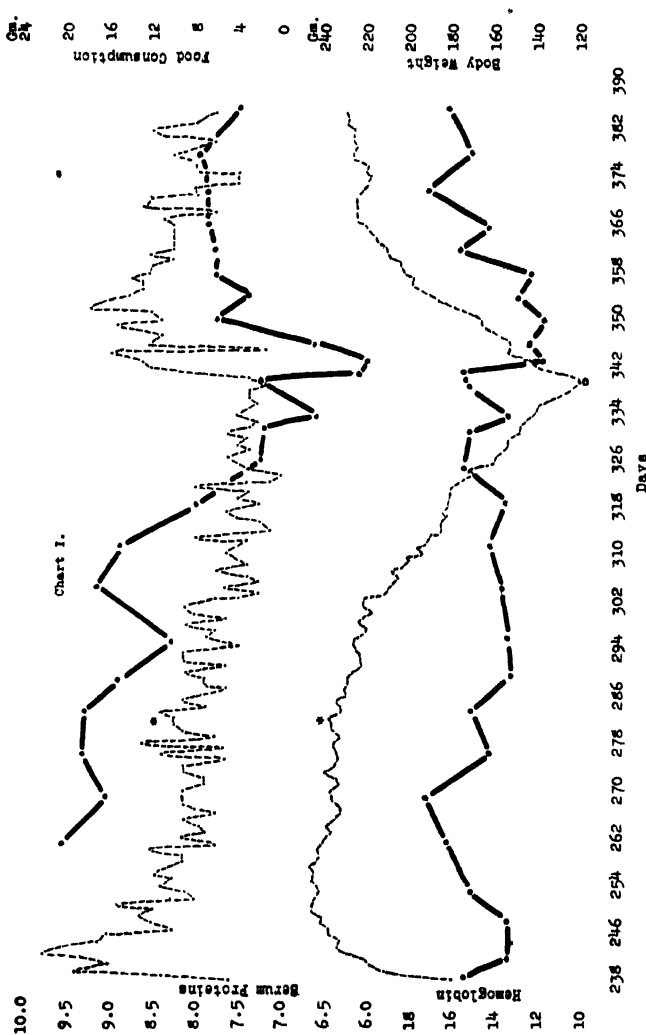
CHART I. ♀ 5156.    For the first 40 days of the experiment this animal received synthetic Ration 1145 containing 10 per cent Northwestern dehydrated yeast as a source of the vitamin B complex.    At point * the animal was given Ration 1009 deficient in the vitamin B complex.    At point □ a supplementary daily allowance was given of 500 mg. of Northwestern dehydrated yeast.    The upper curve in heavy lines represents concentration of serum proteins in per cent The lower curve in heavy lines represents concentration of hemoglobin in gm. per 100 cc. of blood.    The upper curve in dotted lines represents daily food consumption in gm.    The lower curve in dotted lines represents body weight in gm.

abundance of the vitamin B complex.   After that period vitamin depletion was instituted.   Daily records were kept of body weight and food consumption of this as well as of the former group, and hemoglobin determinations and erythrocyte counts were made twice a week.   In the terminal stages of the disease these determinations were carried out three times weekly when necessary whenever time permitted.   We also made total leucocyte counts during various stages of the avitaminosis.   The animals of both groups were killed with chloroform at various stages of the disease and determinations made of the total blood solids and the water content of the tissues.

In Chart I we are presenting an illustration of a typical case, showing a preliminary period of 44 days on an adequate diet (Ration 1145), 55 days of a vitamin depletion period, and 45 days of a subsequent vitamin therapy period.   Records of body weight, food consumption, concentration of hemoglobin and serum protein are shown graphically.   It will be noted that concomitant with the loss in body weight there is a reduction in the concentration of serum proteins.   After vitamin therapy, while the increase in body weight is accompanied by an increase in concentration of serum proteins, the final concentration reached, 8.0 per cent, does not approach the original concentration of 9.3 to 9.5 per cent when the animal was on Ration 1145.   This may have been influenced by the difference in the daily amounts of dehydrated yeast available to the animal during the two periods.   On a daily food intake of 8 to 12 gm. of Ration 1145, 800 to 1200 mg. of yeast were daily accessible to the animal during the first period of the experiment, while during the therapy period only 500 mg. daily of this same yeast were furnished.   The interesting point in this connection is that, although subsequent to vitamin therapy the maximum weight attained is only 4.3 per cent below the maximum weight reached on the 248th day, during the preliminary period on Ration 1145, the maximum concentration of serum proteins was about 16 per cent lower at the termination of the experiment, in spite of the fact that no appreciable differences in food consumption were apparent during these two periods.   The only significant change that was made was the daily proportion of dehydrated yeast allowed.   The first rise in the hemoglobin curve may be attributed to growth produced by the abundance of
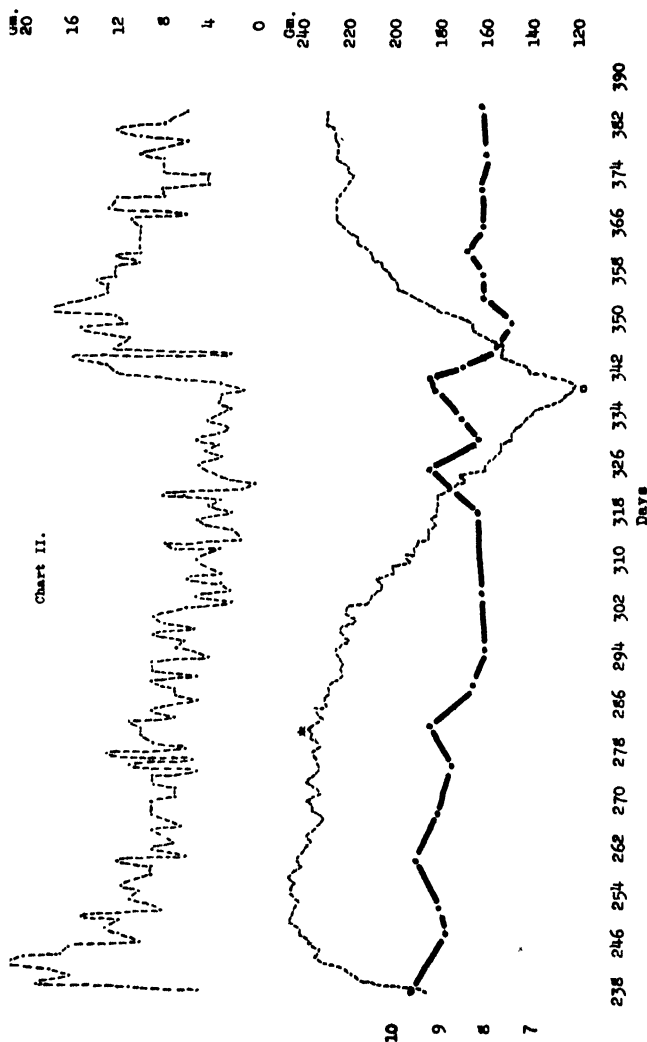
Chart II.



CHART II. ♀ 5156. For the first 40 days of the experiment this animal received synthetic Ration 1145 containing 10 per cent Northwestern dehydrated yeast as a source of the vitamin B complex. At point * the animal was given Ration 1009 deficient in the vitamin B complex. At point □ a supplementary daily allowance was given of 500 mg. of Northwestern dehydrated yeast. The curve in heavy lines represents concentration of erythrocytes expressed in millions per cmm. of blood. The upper curve in dotted lines represents daily food consumption in gm. The lower curve in dotted lines represents body weight in gm.
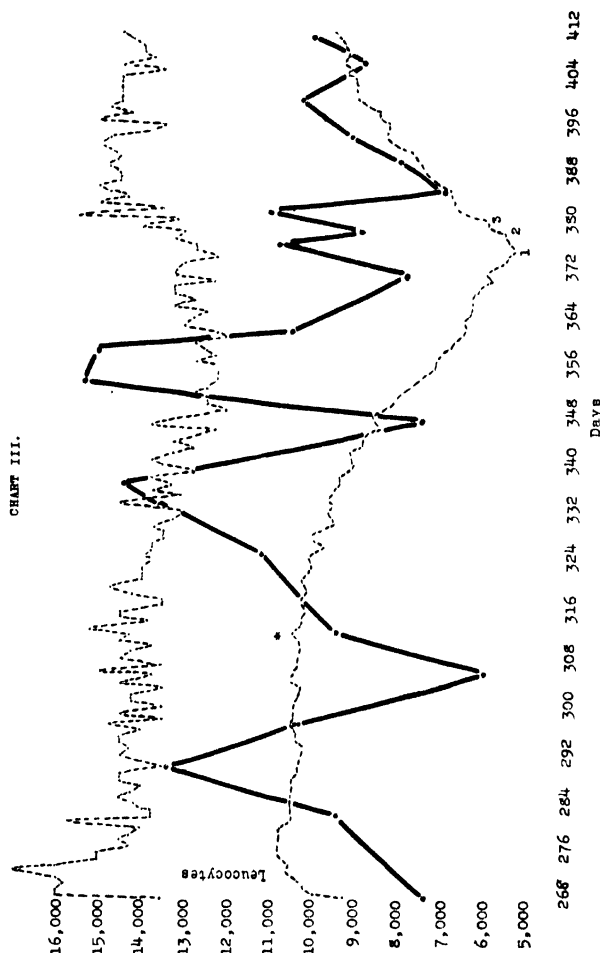
CHART III.

CHART III. ♀ 5153. For the first 40 days of the experiment this animal received synthetic Ration 1145 containing 10 per cent Northwestern dehydrated yeast as a source of the vitamin B complex. At point * this animal was given Ration 1009 deficient in the vitamin B complex. At point "1" a supplementary daily allowance was given of 35 mg. of one of our less potent yeast concentrates (Concentrate 21). At point "2" the daily dosage was increased to 50 mg. At point "3" the yeast concentrate was changed to one of our potent preparations (Concentrate 2), 50 mg. daily being administered until the completion of the experiment. The curve in heavy lines represents leucocytes expressed in thousands per cmm. of blood. The upper curve in dotted lines represents daily food consumption in gm. The lower curve in dotted lines represents body weight in gm.

yeast in the diet. During the first 36 days of the avitaminosis there is a fall in 2 to 3 gm. of hemoglobin per 100 cc. of blood, followed by a rise during later stages of polyneuritis, most probably due to anhydremia, since vitamin therapy is accompanied by a reduction from 15.5 to 11.8 per cent, apparently due to dilution of blood. That the refractive index of the blood serum cannot be relied on to show blood concentration where there is considerable loss of body weight we have recently demonstrated (1). We have, consequently studied this problem on another group of animals, in which prolonged maintenance was produced. These findings will be shown later. The influence of the yeast administration on the increase in concentration of hemoglobin is quite evident. .

In Chart II we are showing the influence of a deficiency of the vitamin B complex on concentration of erythrocytes on the same animal represented in Chart I. It will be noted that there is a reduction of about one million of red blood corpuscles per cmm. during the first 36 days of the vitamin depletion period, which is followed by a marked rise during the later stages of the avitaminosis, and, as in the case of hemoglobin, there is considerable reduction in concentration subsequent to the yeast administration. These fluctuations can also be explained by changes in water metabolism of the blood serum. It will be noted that the concentration of both hemoglobin and erythrocytes reach the highest figures at the period when inanition is most pronounced. Following vitamin therapy there is a definite rise in the concentration of red blood corpuscles, but the final concentration has not paralleled the peak of the hemoglobin curve.

Chart III shows a typical case, out of thirty-nine studied, of the effect of a deficiency of the vitamin B complex on the total leucocytes. From the tremendous fluctuations during the various experimental periods we certainly cannot conclude that a deficiency of vitamins B and G have any influence on the concentration of total leucocytes. Our work in progress, however, seems to show a lymphopenia in polyneuritic nursing young (5). We find no leucopenia as claimed by Happ (6) and Findlay (7).

In Table I are submitted our findings on ten typical individuals that were sacrificed at various stages of the polyneuritis as follows: (a) loss of weight accompanied by no apparent external symptoms;

(b) loss of weight accompanied by posterior paralysis; (c) loss of weight, posterior paralysis, and chills; (d) loss of weight, posterior paralysis, chills, and cyanosis; (e) loss of weight, complete paralysis, shallow respiration, dying; (f) loss of weight, complete paralysis, chills, cyanosis, dying.

In Table II we are showing results on nine females which received, during the postlactation period, vitamin therapy subse-

TABLE I.

*Effect of Deficiency of Vitamin B Complex on Hematopoietic Function of Females during Postlactation Period.*

| Rat No. | Age. | Weight. | Duration of experiment. | Loss of weight. | Total blood solids. | Hb | Erythrocytes. | Serum proteins. | Total leuco-cytes per cmm. | Water content of tissues. | External pathologi-cal symptoms.* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *days* | *gm.* | *days* | *gm.* | *per cent* | *gm. per 100 cc.* | *millions per cmm.* | *per cent* | | *per cent* | |
| 5151 | 355 | 129 | 87 | 45 | 21.5 | 14.15 | 9.48 | 7.09 | 9,600 | 69.0 | n.a.e.s. |
| 5157 | 291 | 153 | 86 | 32 | 19.3 | 15.07 | 7.72 | 7.09 | 11,700 | 68.0 | n.a.e.s. |
| 5138 | 350 | 145 | 106 | 39 | 18.5 | 15.07 | 9.68 | 6.16 | 7,850 | 70.0 | p.p. |
| 5146 | 395 | 145 | 98 | 58 | 20.8 | 16.11 | 8.24 | 7.09 | 12,000 | 67.5 | p.p. |
| 5141 | 381 | 138 | 98 | 66 | 19.3 | 15.56 | 10.72 | 6.39 | 12,000 | 69.6 | p.p., ch. |
| 5142 | 292 | 109 | 100 | 99 | 20.8 | 16.66 | 9.64 | 6.51 | 9,800 | 66.4 | p.p., ch. |
| 5140 | 371 | 122 | 105 | 50. | 17.0 | 13.71 | 7.48 | 5.23 | 13,400 | 67.4 | p.p., ch., c. |
| 5149 | 323 | 127 | 85 | 55 | 24.9 | 15.56 | 9.20 | 8.13 | 6,850 | 67.1 | p.p., ch., c. |
| 5145 | 420 | 123 | 118 | 93 | 21.1 | 15.07 | 9.04 | 7.50 | 5,000 | 67.5 | c.p., r.s., D. |
| 5150 | 311 | 116 | 96 | 99 | † | 12.98 | 9.04 | † | 13,400 | | c.p., ch., c., D. |

* The meaning of the symbols is as follows: n.a.e.s., no apparent external symptoms; p.p., posterior paralysis; c.p., complete paralysis; ch., chills; c., cyanosis; r.s., respiration shallow; D, dying.

† Animal did not yield enough blood for all determinations.

quent to a depletion of the vitamin B complex. Unfortunately, the animals that received vitamin therapy belonged to the first group investigated on which we have not accumulated data on concentration of serum proteins. Our results on twenty-one animals, however, of which ♀ 5156 is a representative case (Table I) during a preliminary period of 44 days on Ration 1145 show a concentration of serum proteins of 8.5 to 9.5 per cent. With these figures as a basis of comparison, all the pathological animals

with the exception of ♀ 5149 show considerable destruction of serum proteins, accompanying loss of body weight.   The average

TABLE II.

*Hematopoietic Function of Females, during Postlactation Period, That Received Vitamin B Therapy Subsequent to Depletion of Vitamin B Complex.*

| Rat No. | Age. | Weight. | Duration of experiment. | Change in weight. | Total blood solids. | Hb | Erythrocytes. | Total leuco-cytes per cmm. | Water content of tissues. | Remarks.* |
|---|---|---|---|---|---|---|---|---|---|---|
| | *days* | *gm.* | *days* | *gm.* | *per cent* | *gm. per 100 cc.* | *mil-lions per cmm.* | | *per cent* | |
| 5153 | 410 | 206 | 142 | −28 | 18.4 | 11.07 | 10.72 | 10,000 | 61.6 | 50 mg. daily of y.c. (Concentrate 2). |
| 5155 | 505 | 177 | 196 | −41 | 18 8 | 16.11 | 8.20 | 8,150 | 63.7 | 15 mg. daily of y.c. (Concentrate 2). |
| 5156 | 385 | 233 | 137 | +11 | 21.5 | 16 11 | 8.12 | 9,700 | 59.7 | 500 mg. daily of N.W. yeast. |
| 4888 | 276 | 205 | 41 | +1 | 19.8 | 14.58 | 10.56 | | 63.0 | 10 mg. daily of y.c. (Concentrate 18). |
| 4897 | 304 | 182 | 69 | −44 | 20.3 | 17.96 | 8.60 | 11,875 | 62.1 | 50 mg. daily of y.c. (Concentrate 2). |
| 4903 | 307 | 210 | 68 | +7 | † | 14.58 | 7.68 | | 64.7 | 25 mg. daily of y.c. (Concentrate 18). |
| 4904 | 183 | 166 | 55 | +6 | 21.2 | 13.34 | 9.20 | | 60.4 | 25 mg. daily of y.c. (Concentrate 2). |
| 4906 | 183 | 176 | 55 | +22 | 21.7 | 14.58 | 10.44 | | 61.8 | "          " |
| 4905 | 183 | 207 | 55 | +29 | 21.2 | 13.71 | 10.80 | | 57.0 | 5 mg. daily of y.c. (Concentrate 16). |

* y.c. indicates yeast concentrate; N.W., Northwestern dehydrated yeast.   For methods of preparation of yeast concentrates see *J. Nutr.*, **1**, 50 (1928).   Yeast Concentrate 2 represents the dehydrated product of the washings of the sodium sulfate crystals obtained by releasing vitamin B from fullers' earth.   All of the above vitamin B preparations were made by E. H. Stuart, chemist of the Research Laboratories of Eli Lilly and Company, Indianapolis.

† Determination lost.

figure for water content of the tissues of the animals that received vitamin therapy is 61.5 per cent, while for the pathological animals the average figure is 68.0 per cent, indicating slight edema in the
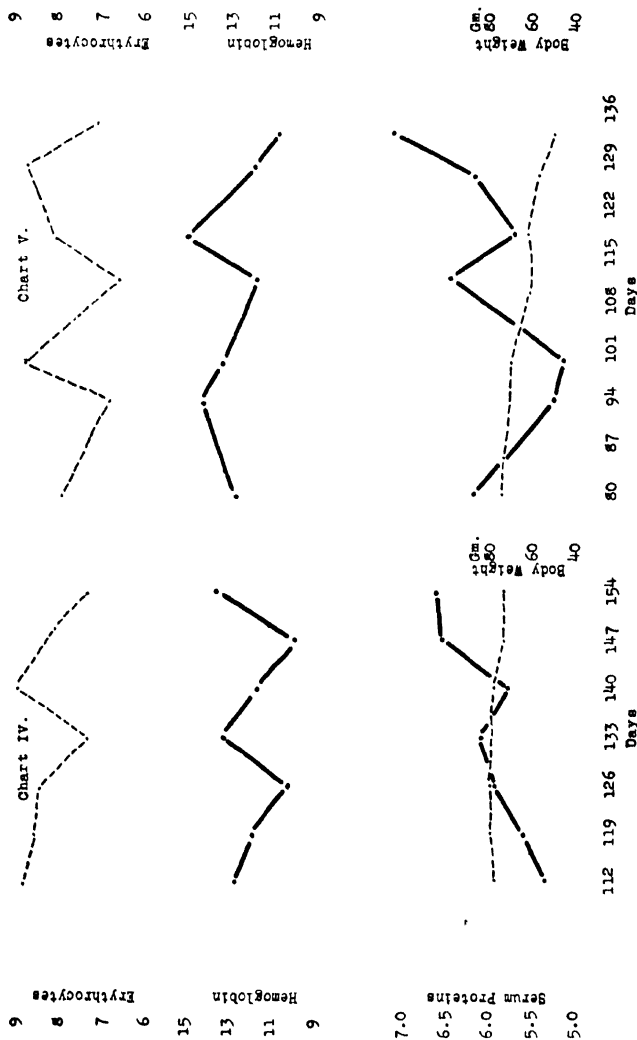
CHART IV. ♀ 1356-b.   This animal throughout the experimental period received Ration 1009 deficient in the vitamin B complex. Maintenance was produced by the daily administration of 3 mg. of yeast concentrate (Concentrate 2) per animal per day. The upper curve in heavy lines represents concentration of hemoglobin expressed in gm. per 100 cc. of blood. The lower curve in heavy lines represents concentration of serum proteins in per cent. The upper curve in dotted lines represents concentration of erythrocytes expressed in millions per cmm. of blood. The lower curve in dotted lines represents body weight in gm.

tissues of the polyneuritic rats. The concentration of total blood solids is the same in the pathological as the control animals, the average figure for both groups being 20.36 per cent. This is possible when the catabolism of serum proteins accompanying heavy losses of body weight takes place to the same extent as the loss of water from the blood serum. In order to demonstrate anhydremia, rapid loss of body weight must be prevented, or preferably prolonged maintenance produced. This we have accomplished and typical cases are submitted in Charts IV and V. The changes in the water content of the tissues with the advance

TABLE III.

*Hematopoietic Function of Lactating Mother during Period When Nursing Young Are Suffering from Uncomplicated Vitamin B Deficiency.*

| Date. | Mother. | | Litter, 6 young. | | Hematopoietic function of mother. | | |
|---|---|---|---|---|---|---|---|
| | Age. | Weight. | Age. | Weight. | Hb | Erythrocytes. | Serum proteins. |
| *1929* | *days* | *gm.* | *days* | *gm.* | *gm. per 100 cc.* | *millions per cmm.* | *per cent* |
| Mar. 22 | 187 | 174 | 20 | 110 | 14.0 | 7.60 | 7 21 |
| " 26 | 191 | 170 | 24 | 114 | 14 5 | 6 92 | 6.80 |
| " 29 | 194 | 160 | 27 | 113 | 16 2 | 7 20 | 5 93 |
| Apr. 2 | 198 | 156 | 31 | 111 | 14 0 | 7.00 | 6 57 |
| " 4 | 200 | 158 | 33 | 111 | 14 7 | 6.40 | 6 27 |

of the avitaminosis are too small to be considered of any importance. The erythrocyte counts and hemoglobin concentration do not show the presence of any anemia. It is also perfectly obvious from Table I that there is no relation between the severity of the polyneuritis and the total leucocyte count.

Another problem we are interested to secure information on is the biochemical changes in the blood of the lactating mother whose young are suffering from uncomplicated vitamin B deficiency because of insufficient vitamin B for optimum lactation. Table III shows a typical case out of six investigated, and clearly demonstrates that during a period of 13 days, when the litter of six young gained only 1 gm., the mother having lost 9 per cent of her body weight, no changes are apparent in the concentration of hemoglobin, a slight reduction in the erythrocyte count is evident,

but a definite reduction takes place in the concentration of serum proteins. In the first part of the paper we have shown, however, that when such mothers that have failed in lactation are given a diet deficient in the vitamin B complex they show marked anemia during intervals of 4 to 10 days. Later on concentration arises due to anhydremia.

*Part II.*—In addition to the thirty-nine adult animals, we have investigated the hematopoietic function of twenty-four animals taken from the mothers at weaning, twelve of which were allowed Ration 1009, deficient in the vitamin B complex, and twelve the same diet, but supplemented with a daily administration per animal of 300 mg. of Northwestern yeast, autoclaved for 6 hours at 15 to 18 pounds pressure. In the latter group we, therefore, developed uncomplicated vitamin B deficiency, the antipellagric factor, vitamin G, having been furnished by the autoclaved yeast.

In the first group that was depleted of the vitamin B complex, prolonged maintenance was produced by the daily administration of 3 mg. of our potent yeast concentrates (Concentrate 2) to each animal. The maintenance undoubtedly was partly influenced by the vitamin B that was stored from the previous dietary régime, which allowed large dosages of yeast concentrates daily to the litters during the period of lactation. In Chart IV is presented a typical case. The marked rise in the concentration of serum proteins during 42 days in which there was no growth unmistakably shows anhydremia. There was also disturbance in hematopoietic function, as evidenced by fluctuations in concentration of hemoglobin and erythrocytes.

In Chart V we are submitting a representative individual showing the effect of uncomplicated vitamin B deficiency on hematopoietic function. The marked increase in the concentration of serum proteins during the period the animal is gradually losing weight undoubtedly establishes anhydremia. As is the case of a deficiency of the vitamin B complex, there is an accompanying disturbance in hematopoietic function indicated by fluctuations in the concentration of hemoglobin and red blood corpuscles. There is no evidence of a definite anemia.

Food consumption records show that the animals of both groups were eating approximately 32 gm. weekly, or 4.5 gm. daily, or about 50 to 60 per cent of what is consumed by an animal of that

weight and age on an adequate diet containing an abundance of vitamins B and G.  Our conclusions, then, are that undernutrition is responsible for such a severe pathological symptom as anhydremia, which is the condition noted by Marriott (8) in the last stages of marasmus in infants.  We, therefore, do not agree with Drummond and Marrian (9) that the pathological changes produced by vitamin B deficiency (complex) is nothing more than a manifestation of the effects of starvation.  To be sure, during the last stages of polyneuritis the inanition is so marked as to approach starvation, but we encountered anhydremia during the earlier stages of the disease, which must be attributed to *underfeeding* rather than to *starvation.*

The character of our results certainly does not show any relation between a deficiency of the vitamin B complex or uncomplicated vitamin B deficiency and pernicious anemia, or, as a matter of fact, does not establish the association with any definite form of anemia.  The fluctuations in concentration of hemoglobin and erythrocytes, and the reduction in concentration of serum proteins, and the anhydremia encountered, justify the conclusion that during polyneuritis there is produced a disturbance in hematopoietic function.

### SUMMARY.

A deficiency of the vitamin B complex produces the following pathological symptoms.

1. Reduction in the concentration of serum proteins when accompanied by heavy losses in weight.

2. Disturbance in hematopoietic function as evidenced by fluctuations in concentration of erythrocytes and hemoglobin. During the early stages of the avitaminosis there is a small reduction in the concentration of hemoglobin and red blood corpuscles, and during the final stages of the disease there is a marked rise in both of these constituents.  This rise is associated with marked anhydremia produced by pronounced inanition.

3. When loss of body weight is circumvented and prolonged maintenance produced by feeding suboptimum amounts of vitamins B and G in the form of yeast concentrates, loss of water from the blood serum, or anhydremia, becomes apparent.

4. There is a slight edema of the tissues in polyneuritis to the extent of about 6.5 per cent.

5. The avitaminosis has no specific effect on the total leucocyte count.

6. Uncomplicated vitamin B deficiency also produces disturbance in hematopoietic function associated with anhydremia, the latter pathological condition being only apparent during stages of prolonged maintenance.

7. Mothers whose young are developing uncomplicated vitamin B deficiency, because of suboptimum amounts of vitamin B for normal lactation, show no change in the concentration of hemoglobin, slight reduction in the concentration of erythrocytes, but a notable reduction in the concentration of serum proteins.

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# THE EFFECT OF AVITAMINOSIS ON HEMATOPOIETIC FUNCTION.

## III. VITAMIN E DEFICIENCY.*

By BARNETT SURE, M. C. KIK, and DOROTHY J. WALKER.

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Stimulated by the suggestion of Hart and coworkers (1) Hogan and Harshaw (2) were the first to investigate the possible rôle of vitamin E in hematopoietic function, and in 1926 reported negative results. They showed that the erythrocyte counts of females that resorbed their fetuses because of vitamin E deficiency were no different from animals that carried their litters to term on adequate diets containing an abundance of the reproductive factor. The authors also stated that they encountered no reduction in the concentration of hemoglobin in either the males or females on the vitamin E-deficient rations, but no figures on hemoglobin have been submitted.

In 1927, Simmonds, Becker, and McCollum (3), in their study on the "Relation of Vitamin E to Iron Assimilation" first concluded: "The function of vitamin E is in some manner associated with iron assimilation." Their conclusion was based on the fact that they were able to cure a so called "salt-ophthalmia," associated with decline in body weight, either by changing the ferrous sulfate in their salt mixture to ferric sulfate, or by the addition of wheat germ oil (the most potent source of vitamin E) to their diets. They further stated: "Our work offers a new interpretation for certain experimental data of other investigators who have attributed pernicious anemia to lack of vitamin A, and suggests a new point of view in interpreting the cause of sterility attributable to faulty nutrition." In the absence of data on hemoglobin determinations or erythrocyte counts the conclusions

of Simmonds, Becker, and McCollum were not convincing to us. About the time their communication appeared Hart and coworkers (4) demonstrated the correction of a nutritional anemia on whole milk diets with the ash of certain plant and animal tissues, and it

TABLE I.

*Composition of Rations.*

| Ration No | Casein * | Salts 32 † | Salts 185 ‡ | Ferric citrate | Agar-agar | Northwestern yeast | Brewers' yeast | Acetone-extracted wheat embryo | Cod liver oil § | Lard | Wheat germ oil | Dextrin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | cc | | | |
| 1212 | 20 | 4 | | | | | 10 | | 0 3 | 1 | | 65 0 |
| 1213 | 20 | 4 | | | | | 10 | | 0 3 | | 1 | 65 0 |
| 1214‖ | 20 | 4 | | | | | 10 | | 0 3 | 1 | | 65 0 |
| 1215 | 20 | 4 | | 0 2 | | | 10 | | 0 3 | 1 | | 64 8 |
| 1216 | 20 | 4 | | 0 2 | | | 10 | | 0 3 | | 1 | 64 8 |
| 1217‖ | 20 | 4 | | 0 2 | | | 10 | | 0 3 | 1 | | 64 8 |
| 1296 | 20 | 4 | | 0 2 | | 10 | | | 0 3 | | 3 | 62 8 |
| 1297 | 20 | 4 | | 0 2 | | 10 | | | 0 3 | | 1 | 64 8 |
| 1298 | 20 | | 4 | | 2 | 10 | | | 0 3 | 5 | | 59 0 |
| 1299 | 20 | | 4 | | 2 | 10 | | | 0 6 | 5 | | 59 0 |
| 1300 | 20 | | 4 | | 2 | 10 | | | 0 3 | 5 | 0 3 cc daily. | 59 0 |
| 1302 | 20 | | 4 | | 2 | | | 30 | 0 3 | | 0 3 "   " | 43 0 |
| 1303 | 20 | | 4 | | 2 | | | 30 | 0 3 | 3 | | 43 0 |
| 1322 | 20 | 4 | | | | | | 30 | 0 3 | | | 46 0 |
| 1323 | 20 | 4 | | | | | | 30 | 0 3 | 3 | | 43 0 |
| 1324 | 20 | 4 | | | | | | 30 | 0 3 | | 3 | 43 0 |
| 1325 | 20 | 4 | | | | | | 30 | 0 6 | 3 | | 43 0 |
| 1326 | 20 | 4 | | | | | | 30 | 0 3 | 3 | 0 3 cc. daily. | 43 0 |

\* Purified by washing for a week with dilute acetic acid.

† Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, **40**, 501 (1919).

‡ McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

§ Daily except Sundays.

‖ 180 mg. of ash of lettuce leaves daily to each animal.

was suggested by Professor Hart to the senior author to carry out experiments with the ash of lettuce leaves as a substitute for vitamin E, in order to determine whether some inorganic factor other than iron is associated with vitamin E physiology.   Professor Hart supplied us with liberal amounts of the ash of lettuce leaves, and we

began the investigation the early part of October of 1927.   In the meantime, Mattill (5) demonstrated that certain vegetable oils, especially wheat oil, delay autooxidation in fats and thereby prevent accompanying destruction of vitamins A and E.   Simultaneous with the appearance of Mattill's paper, Jones (6) produced evidence that if the butter fat in the rations of McCollum, Simmonds, and Becker (7) were incorporated at intervals of about

TABLE II

*Fertility Record*

| xperiment No | Duration of experiment | Reproduction period | No of litters | Total No of young born | Total No of young born alive | Resorptions, postmortem |
|---|---|---|---|---|---|---|
| | days | days | | | | |
| 1212 | 148 | 71 | 0 | 0 | 0 | 1 |
| 1213 | 237 | 158 | 10 | 60 | 56 | 0 |
| 1214 | 203 | 122 | 3 | 15 | 13 | 3 |
| 1215 | 233 | 123 | 2 | 12 | 6 | 2 |
| 1216 | 259 | 151 | 9 | 70 | 69 | 0 |
| 1217 | 211 | 101 | 1 | 8 | 6 | 4 |
| 1296 | 191 | 105 | 8 | 63 | 58 | 0 |
| 1297 | 191 | 105 | 8 | 71 | 69 | 0 |
| 1298 | 156 | 70 | 0 | 0 | 0 | 4 |
| 1299 | 188 | 105 | 2 | 11 | 1 | 1 |
| 1300 | 217 | 133 | 6 | 50 | 50 | 0 |
| 1302 | 235 | 150 | 7 | 37 | 35 | 0 |
| 1303 | 188 | 71 | 0 | 0 | 0 | 0 |
| 1322 | 201 | 100 | 4 | 20 | 20 | 1 |
| 1323 | 201 | 99 | 2 | 13 | 11 | 0 |
| 1324 | 224 | 117 | 6 | 56 | 56 | 0 |
| 1325 | 189 | 94 | 3 | 17 | 10 | 0 |
| 1323 | 212 | 117 | 5 | 33 | 31 | 0 |

5 days, the animals grew normally and did not develop sore eyes. About the same time, McCollum, Simmonds, and Becker (8) reported similar findings and recently have changed their interpretations of the influence of the addition of wheat germ oil to their "salt-ophthalmia" diets (9), stating: "The injury due to ferrous sulfate is the result of destruction of vitamin A.   There is no evidence that vitamin E has any rôle in iron assimilation."

During the progress of this investigation Hart and coworkers (10) have demonstrated that copper is the inorganic element in

TABLE III.

*Effect of Vitamin E Deficiency on Hematopoietic Function.*

Hb = hemoglobin expressed in gm. per 100 cc.
RBC = red blood corpuscles expressed in millions per cmm.
TL = Total leucocytes expressed in figures per cmm.

| Ration No. | Animal No. | Age | Weight | At breeding | | | Advanced stage of pregnancy. | | | Sterility period (no apparent signs of resorption). | | | Period of resorption of fetus. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Hb | RBC | TL | Hb | RBC | TL | Hb | RBC | TL | Hb | RBC | TL |
| | | days | gm. | | | | | | | | | | | | |
| 1212 | 4923 | 125 | 161 | 12 98 | 8 44 | 8,250 | | | | | | | | | |
| | | 147 | 176 | | | | | | | 13.34 | 9.72 | 9,150 | | | |
| | | 173 | 202 | | | | | | | 14.15 | 9.36 | | | | |
| 1213 | 4929 | 126 | 176 | 12 30 | 9 08 | 10,925 | | | | | | | | | |
| | | 147 | 191 | | | | | | | 13.34 | 8 52 | 9,175 | | | |
| | | 171 | 221 | | | | 13.71* | 8.00* | | | | | | | |
| | | 200 | 233 | | | | 12 98† | 7.16† | 11,600† | | | | | | |
| 1214 | 4935 | 128 | 162 | 18.7 | 8.24 | 13,150 | | | | | | | | | |
| | | 149 | 145 | | | | | | | 15.56 | 10.48 | 7,000 | 16.66 | 9.96 | 15,950 |
| | | 151 | 136 | | | | | | | | | | | | |
| 1215 | 4942 | 131 | 164 | 18.7 | 8.20 | 8,450 | | | | | | | | | |
| | | 151 | 166 | | | | | | | 12.61 | 9.48 | 8,150 | | | |
| | | 182 | 194 | | | | | | | 13.34 | 10.20 | | | | |
| | | 201 | 204 | | | | 15.07 | 7.68 | 13,350 | | | | | | |
| | | 209 | 232 | | | | 15.56 | 8.64 | 12,050 | | | | 13.34 | 6.72 | 14,050 |
| | | 212 | 214 | | | | | | | | | | | | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1216 | 4949 | 130<br>154<br>165<br>193 | 188<br>195<br>251<br>248 | 14 15 | 8 96 | 17,050 | 13 34*<br>12 30† | 9 24*<br>8 04† | 8,200 | 12 30 | 8 92 | 7,100 | 14 58 | 7 88 | 10,350 |
| 1217 | 4953 | 137<br>146<br>176<br>196<br>200 | 205<br>200<br>204<br>222<br>213 | 12 61 | 9 88 | 10,125 | 15 56 | 8 32 | 10,150 | 13 34<br>12 96 | 9 56<br>8 40 | 15,156 | • | | |
| 1322 | 5251 | 111<br>131<br>144<br>170 | 187<br>207<br>250<br>218 | 13 71 | 8 04 | 11,800 | 13 71‡<br>12 30† | 8 48‡ | 10,600‡ | 9 53 | 9 08 | 9,850 | | | |
| 1324 | 5261 | 114<br>133<br>221 | 188<br>216<br>262 | 13 71 | 9 36 | 12,550 | 13 34*<br>12 30† | 7 80*<br>7 80† | 11,650*<br>8,560† | | | | 16 11§ | 9 42§ | 9,200§ |

* Pregnancy resulting in first litter.
† Pregnancy resulting in second litter.
‡ Had a first litter of four young which died the day after delivery.
§ Resorption after second mating.

the ash of plant materials that acts as a supplement to iron in the synthesis of hemoglobin by the albino rat.   Although our findings on the rôle of vitamin E in hematopoietic function are negative, we believe they are of interest because they offer additional evidence that neither a deficiency of iron nor of other inorganic elements contained in the ash of lettuce leaves has any relationship to the resorption of the fetus during gestation produced by vitamin E deficiency.

Our results are summarized in Tables I to III.   For each type of diet we used two males and four females, which, for the eighteen rations, makes a total of thirty-six males and 72 females studied. Weekly records were kept of food consumption, but on the first external signs of advanced pregnancy the females were separated in individual compartments and daily records taken of food intake. Our data disclosed the fact that during the last few days of pregnancy of the animals receiving vitamin E-containing diets there is a notable reduction in food consumption, in some cases a decrease from 10 to 14 gm. daily to 7 and 5 gm. daily.   Similar findings were obtained with the majority of the animals that showed typical resorption curves produced by vitamin E deficiency, the resorptions being confirmed by postmortem examinations of the uterine horns.   A dozen of the females ate 10 to 12 gm. daily during 2 to 3 days of the resorption period, only one having shown a marked reduction in food intake to 2 gm. daily.   All the animals made excellent growth on the diets indicated in Table I, the food consumption varied weekly from 65 to 95 gm. among the animals on the vitamin E-deficient diets, the same extent of variation being noted among the controls.   The food records are of no special interest in this investigation and are, therefore, deleted.   The diets abundant in vitamin E are either those containing wheat oil in the ration or those which were supplemented by separate wheat oil administrations.   The thirty-two females on the eight vitamin E-containing rations had 59 litters of 440 young, 424 of which were born alive; while the forty females on the ten vitamin E-deficient rations had sixteen resorptions (determined by postmortem examination) and seventeen litters of 86 young, 67 of which were born alive.

Hemoglobin determinations and erythrocyte and total leucocyte counts were made on each animal once weekly from the time of

mating until the period of advanced pregnancy. From that stage on at least two readings were obtained until the litter was delivered at term, or until the animal was sacrificed for the determination of resorption (indicated by the resorption curve of daily records of body weight) (11). We have also made similar determinations for a period of several months at least once weekly on those females that showed no apparent signs of pregnancy. In Table III we are showing eight representative cases which clearly prove that during the period of resorption of the fetus the concentration of hemoglobin and erythrocytes is no different from that found at the time of mating or that manifested during advanced pregnancy resulting in the birth of normal litters of young. We also found no influence of vitamin E deficiency on the total leucocyte count. It is also interesting to point out in this connection that we have not observed an anemia with the advance of pregnancy on the types of diets used in this study, which are characterized mainly by the abundance of either dehydrated yeast or defatted wheat embryo, as encountered previously on our standard cereal ration, Stock Diet 1 (12).

The fortification of our vitamin E-deficient rations with additional amounts of ferric citrate did not prevent resorption of the fetus (Ration 1215), neither did the addition of 180 mg. of the ash of lettuce leaves per animal per day,[1] in the absence or presence of additional amounts of ferric citrate, prevent female sterility produced by vitamin E deficiency (Rations 1214 and 1217). We conclude, therefore, that there is no association between iron or other mineral constituents of the ash of lettuce leaves and vitamin E function.

### SUMMARY.                           .

1. Vitamin E has no influence on hematopoietic function of females suffering from such avitaminosis during the period of resorption of the fetus.

2. Neither ferric citrate nor the ash of lettuce leaves were found effectual in the prevention of female sterility produced by vitamin E deficiency.

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[1] This daily dosage of the ash of lettuce leaves was recommended by Professor Hart, and was fed mixed with the yeast separately from the ration.

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# ENDOGENOUS URIC ACID AND HEMATOPOIESIS.

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Following the classification of Burian and Shur (1900), two sources of uric acid in the blood and urine are generally recognized; the exogenous, or that resulting from the purines of the food, and the endogenous, or that derived from tissue metabolism. The exact origin of the latter, however, is not known, nor is the rôle of the uric acid in the body economy established.

The more generally accepted view of the origin of endogenous uric acid is that of nuclear disintegration. This is based on the initial observation of Horbaczewski (1887) that uric acid may be produced *in vitro* from splenic pulp, secondly, that uric acid is increased upon the ingestion of tissues rich in nuclear material such as liver, pancreas, and thymus (Umber, 1896); that nucleic acid contains two purine components (Levene and Jacobs, 1912); and that all of the enzymes necessary for the destruction of nucleic acid are present in the body (Jones, 1920).

Burian and Shur (1900), however, oppose this theory on the ground that Horbaczewski's assumption of leucocyte destruction failed to account quantitatively for the results in disease, and also failed to explain the practically constant level established for every individual in health. They concluded that uric acid was an end-product of muscle metabolism derived from the hypoxanthine of the acid in birds, and by the partial recovery of hypoxanthine as uric acid after injection. Siven (1901), however, was unable to demonstrate a correlation between muscular exercise and uric acid output. Kennaway (1909) showed a gradual decrease of output with a repetition of equivalent exercise on successive days.

Mares (1910) attempted to account for the endogenous uric acid on the basis of increased "wear and tear" or activity on the part of the digestive cells, since it was shown by Folin (1905) that a pro-

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tein diet increased the uric acid output.   This interpretation has been questioned by Lewis, Dunn, and Doisy (1918) on the basis of their observations of an increase in hourly output after the administration of amino acids, and the observation of Smetanka (1911) that there is also an increase after the ingestion of honey, which requires little or no digestion.   Furthermore, Zwarenstein (1928) found no increase after the ingestion of 200 gm. of Cheddar cheese or boiled egg white.   Gibson and Doisy (1923) showed that while pyruvic acid increased the uric acid output, lactic acid decreased it.

Graham and Poulton (1913), observing a fall in uric acid on a protein-fat and a carbohydrate-fat diet, advanced an explanation involving a balanced carbohydrate-protein synthesis.   •

These various hypotheses, including the one of a general cell stimulation dependent upon the calorific values of the food taken in, are rendered untenable by the observation of Lennox (1924) who has shown a marked increase in uric acid output during periods of prolonged fasting.   For a general review of the subject reference may be made to the papers of Benedict (1916), Rose (1923), and Folin, Berglund, and Derick (1924).

### Theoretical Considerations.

On the hypothesis that endogenous uric acid may arise from the extruded nuclei of the normoblasts at the maturation of the erythrocytes, a series of calculations was made dealing with the quantitative relations of the substances involved.   The results indicate a large and constant source of uric acid sufficient in amount to account for the observed outputs.

The total blood volume of man is generally placed at 5.5 liters (Keith, Rowntree, and Geraghty, 1915).   The corpuscles represent 35 per cent of this by volume, or 1,925 cc.   Blood destruction rate, based on the bilirubin output, is placed at 6.6 per cent by Brugsch and Yoshimoto (1910), 2.9 per cent by Eppinger and Charnas (1913), and 1.5 per cent by Abderhalden.   Based upon this, the lowest estimate, the total volume of red corpuscles destroyed daily would be 28.9 cc. or 31.4 gm.   Since the red blood cell count remains practically constant over long periods of time, the rate of production must equal the rate of destruction.

Measurements on the size of the pycnotic normoblast nucleus at the time of extrusion give an average diameter of $5\mu$, and an estimated volume of 65.44 cubic $\mu$. This is approximately equal to the volume of the erythrocyte with an estimated value of 71 cubic $\mu$. This would then indicate that 28.8 gm. of nuclear material are available for destruction daily. Of the chromatin of this pycnotic nucleus approximately half is nucleic acid, or 14.4 gm. Nucleic acid yields both adenine and guanine, which thus provide 2 molecules of uric acid. Uric acid with a molecular weight of 213 is 14.7 per cent of the nucleic acid molecule (molecular weight, 1443) (Levene and Jacobs, 1912). 4.23 gm. of uric acid may thus be theoretically produced daily from the destruction of the extruded nuclei of the normoblasts in the process of erythrocyte formation. This figure is of interest when compared to the amount of 0.3 gm. to which Folin was able to reduce his uric acid output on a purine-free diet. It would still be in excess if it be considered that the nucleus contained 50 or 75 per cent water, or that adenine and guanine might be reutilized in nuclear construction in adjacent cells.

### Experimental Methods and Results.

In order to test the hypothesis of a relationship between endogenous uric acid and hematopoiesis, a thoroughbred Dalmatian coach dog was obtained from a reliable kennel; the uric acid level was established over a period of 12 days; the dog was then bled and the posthemorrhagic level determined. The dog was a young male, born July 24, 1928. He weighed at the time of bleeding, 15 kilos. He was kept in an animal cage and the urine was collected through a floor drain. Toluene was used as a preservative. The specimens were collected every morning at 9 a.m. At that time the dog was taken from the cage, while the latter was thoroughly cleaned. He was returned to the cage and given a light breakfast of eggs, grits, or Wheatena. At 1 p.m. he was given a fairly heavy meal of freshly cooked beef, and vegetables, such as rice, carrots, peas, cowpeas, cabbage, or turnip greens. The first 3 days he was on beef and dog biscuit, but this proved too constipating. No trouble was had after the freshly cooked vegetables were substituted for the dog biscuit. The animal remained in excellent condition throughout the experimental period.

Uric acid determinations were made by the Benedict-Franke method. The values of the prehemorrhagic period varied from 66.6 to 256 mg. with an average of 154 mg. for the 12 days. The dog was bled April 30 at 4 p.m. by Dr. Ralph Chaney of the Department of Surgery. 350 cc. of blood were withdrawn from the femoral artery under local anesthesia. This represents 2.5 per

TABLE 1.

*Showing Effect of Hemorrhage on Uric Acid Output of Dalmatian Dog.*

|  | Total urine. | Uric acid. |  |  | Total urine | Uric acid. |
|---|---|---|---|---|---|---|
|  | *cc.* | *mg.* |  |  | *cc.* | *mg.* |
| Apr. 19 | 440 | 130 | May 1 | | 450 | 129 |
| 20 | 1500* | 171 | | 2 | 700 | 466 R.B.C. 3,300,000 |
| 21 | 1350* | 256 | | 3 | 600 | 320 |
| 22 | 600 | 200 | | 4 | 700 | 233 |
| 23 | 240 | 87 | | 5 | 650 | 260 |
| 24 | 200 | 66 | | 6 | 640 | 320 R.B.C. 4,010,000 |
| 25 | 660 | 136 | | 7 | 700 | 330 |
| 26 | 450 | 90 | | 8 | 660 | 293 |
| 27 | 550 | 191 | | 9 | 720 | 288 |
| 28 | 590 | 139 | | 10 | 800 | 266 |
| 29 | 920 | 193 | | 11 | 550 | 275 |
| 30 | 520 | 181 | | 12 | 1300 | 346 |
| Average . . . . . | | 154 | | 13 | 480 | 240 R.B.C. 6,360,000 |
| | | | | 14 | 520 | 346 |
| | | | | 15 | 750 | 250 |
| | | | Average . . . . . . . . | | 302† |

After readings for Apr. 30 were taken the dog weighed 15 kilos and was bled 350 cc. at 4 p.m.

* Increased volume due to dilution by drinking water.

† Exclusive of the 129 mg. of May 1.

cent of the total body weight and approximately one-third of the total blood volume. The animal withstood the operation readily and ate a hearty meal at 5 p.m. The uric acid determinations for the succeeding 15 days are given in Table I.

If the value for the 1st day be excluded, and it very evidently belongs to the prehemorrhagic period, the average for the posthemorrhagic period is 302 mg. In an extensive analysis of the relation of regeneration to hemorrhage, Dawson (1901) has shown that approximately half of the number of erythrocytes

removed by severe hemorrhage, are replaced between the 4th and 15th day. Thus with an estimated 33 per cent removal and 50 per cent of this amount replaced, the average daily replacement would be approximately 1 per cent. On the hypothesis of one and one-half per cent destruction and replacement under normal conditions, the uric acid levels before and after bleeding are markedly consistent. The high posthemorrhagic level represents the normal 1.5 per cent plus an additional 1 per cent due to compensation.

The extremely high level on the 2nd day is of interest, since it precedes the increase in reticulocytes as ordinarily observed following hemorrhage. It is further indicative of a marked stimulation of the bone marrow while the subsequent sharp drop·would indicate a rapid fatigue.

<center>DISCUSSION.</center>

While the hypothesis of the production of endogenous uric acid from the extruded nuclei of the normoblast is supported by a single experiment only, the results are clear cut and fit the theoretical considerations. Food ingestion can hardly account for the results, since the animal was given the same diet throughout the experiment. Exercise is not a factor since the animal was confined to a cage, free to move, but limited in activity. With a direct correlation between erythrocyte count and uric acid output, the results receive their simplest explanation on the foregoing hypothesis.

That erythropoiesis is the only factor in the production of uric acid is of course not shown. A nuclear metabolism for all tissues is generally assumed, but nuclear destruction in quantities sufficient to account for the daily output of uric acid in normal conditions has no histological support, except in the instance of the bone marrow.

In disease, the increase in uric acid in leucemia, anemia, lead poisoning, and pneumonia may be accounted for in the erythrocyte behavior.

The high uric acid output by the new-born (Schloss and Crawford, ·1911), the increase during pregnancy (Slemons and Bogert, 1917), the return to a lower level after parturition (Williams, 1921), and the increased variations during menstruation (Okey and Erikson, 1926) are suggestive of the soundness of the hypothesis.

SUMMARY.

1. The average daily output of uric acid for a Dalmatian coach dog for a period of 12 days was 154 mg.

2. The average daily output of uric acid for the same dog on the same purine-free diet, but after the removal of 350 cc. of blood, was 302 mg., for the first 15 days, exclusive of the 1st day.

3. This increase in the output of uric acid after hemorrhage is considered to arise from the increased activity of the hematopoietic tissue in producing red blood cells, with the concomitant destruction of the nuclei of the normoblasts.

4. The source of endogenous uric acid from the nucleic acid of the normoblast nuclei is indicated on experimental and theoretical grounds.

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# THE DETERMINATION OF ACETONE BODIES IN BLOOD AND URINE.

## REPLY TO CRITICISMS BY E. C. SMITH.

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(Received for publication, June 8, 1929.)

Methods for determination of the "acetone bodies," acetone, acetoacetic acid, and $\beta$-hydroxybutyric acid, have been published by the writer (Van Slyke, 1917), based upon Deniges' procedure for precipitating acetone as a basic mercuric sulfate compound weighing approximately 20 times as much as the acetone it contains. Hydroxybutyric acid was oxidized to acetone by the chromate oxidation introduced by Shaffer (1908–09). Oxidation and precipitation were carried out simultaneously, so that the technique for determining the total acetone bodies consisted merely in boiling properly prepared urine or blood filtrate (Van Slyke and Fitz, 1917, 1919) with a specified mixture of sulfuric acid, mercuric sulfate, and dichromate under a reflux condenser, the resultant precipitate being either weighed or redissolved and determined by titration of its mercury according to Personne's method. Acetone and acetoacetic acid, without the hydroxybutyric acid, could be determined separately by omitting the chromate from the reaction mixture.

Glucose and most interfering substances occurring in normal or pathological urine were removed by a preliminary precipitation with cupric sulfate and calcium hydroxide. A trace was found to remain of non-acetone substances which precipitate with the mercuric sulfate. For these substances a correction was described; the acetone was distilled off from the clarified urine and the residual solution was boiled with mercuric sulfate without chromate. In a series of twenty-three normal urines the total acetone bodies determined without subtracting the correction for these interfering substances was in all cases below 0.05 per cent, calculated as acetone. Corrected for the non-acetone precipitate the total acetone bodies in all cases were below 0.03 per cent. Ketone-free diabetic urines yielded similar values. In a series of ketone-containing urines the method for hydroxybutyric acid was compared with that of Black (1908–09), in which the acidified urine is mixed with plaster of Paris, the hydroxybutyric acid is extracted with ether, and estimated polarimetrically by its levorotation. Black's method gave 85 to 100 per cent as much hydroxybutyric

acid as the writer's.   Considering that, as previously pointed out by Shaffer and Marriott (1913–14), the polarimetric results are likely to be somewhat low, because of adsorption of hydroxybutyric acid by the charcoal used in preliminary clearing of the urine, because of slight racemization of the acid during extraction, and because of incomplete extraction, it was considered that the agreement was satisfactory.  From the comparison with Black's method in ketone-containing urines and from the nearly negative results with ketone-free urines, it appeared that the new method could be trusted to give complete yields of the acetone bodies present, and to be free from significant error due to interference by non-ketone urinary constituents. Since its appearance the method has received fairly extensive use over periods of years in a number of laboratories, and has apparently met these requirements.

Smith (1926), however, has recently criticized the procedure, for both blood and urine, on the following grounds.

1. In blood used for perfusion experiments he found that the amount of lactic acid present (of the order of magnitude of 100 mg. per 100 cc.) could increase significantly the yield of acetone-mercuric-sulfate precipitate obtained in determination of the total acetone bodies.   Smith ignores the fact that the behavior of lactic acid was pointed out with quantitative data, on p. 486 of the writer's original paper (1917).   It was there shown that lactic acid when boiled with chromate under the conditions of the analysis yields some product which precipitates with mercuric sulfate.   The amount of precipitate formed per mg. of lactic acid was found, however, to be only about one-twentieth of that formed per mg. of acetone.   Blood freshly drawn from the circulation has not enough lactic acid to cause a significant error, except in comparatively rare specimens, such as are drawn immediately after severe exercise or asphyxia.   As shown by Evans (1922), however, if drawn blood is allowed to incubate for some hours its glucose is in large part converted into lactic acid.   This conversion appears to be especially rapid with dog blood, compared with human and horse bloods.   The effect of the amount of lactic acid in the blood used in Smith's perfusion experiments could have been foretold from data in the writer's original paper.

In ordinary blood analyses the effect is insignificant, as shown by the fact that only 1 or 2 mg. of precipitate are obtained from the filtrate of 5 cc. of normal blood (Van Slyke and Fitz, 1917).   To yield an amount of precipitate equal to that from acetone bodies

in a concentration of 1 millimol per liter of blood, about 110 mg. of lactic acid per 100 cc. of blood would be required. Normal, diabetic, and most pathological blood drawn during rest or ordinary activity contains only 10 to 40 mg. per 100 cc. (Clausen, 1922; Ronzoni and Wallen-Lawrence, 1927). After severe exercise it may rise to over 100 mg., likewise in cardiac decompensation sufficient to result in severe anoxemia (Meakins and Long, 1927). In these conditions the lactic acid may cause 5 to 10 mg. of precipitate in the total acetone bodies determination in blood, enough to simulate a slight ketosis, sufficient to be detectable, but not sufficient to be important in the acid-base balance of the blood.

2. Smith states that with diabetic urine the writer's method for total acetone bodies (acetone plus acetoacetic acid plus β-hydroxybutyric acid) yields results which are markedly too high. The basis for this conclusion was that when the precipitate was redissolved in 10 per cent hydrochloric acid, and the acetone was distilled therefrom and titrated with iodine, only 72 per cent as much acetone was indicated by the titration as was expected on the assumption that the precipitate consisted of 5 per cent by weight of acetone. When Smith applied the same procedure to the mercury precipitate from a pure acetone solution, the titration indicated the amount of acetone estimated from the weight of the precipitate. In reporting the low yield of acetone obtained by distillation from the urine precipitate Smith gives no details: it is impossible to tell whether he gives the result of a single observation or the mean of a number, whether the urine analyzed contained much or little acetone, and whether correction was made, as directed in the writer's original paper, for the slight weight of precipitate formed by the mercuric sulfate with non-acetone urinary constituents. The precipitate from such substances is only 1 or 2 per cent of the total precipitate obtained in analyses of urines from subjects with severe or moderate ketosis, but is more important if the ketosis is slight, and in normal urine may exceed the precipitate yielded by the acetone bodies themselves. These facts are illustrated by the series of analyses of diabetic and normal urines published in the original paper (Van Slyke, 1917). Smith limited his control analyses to pure acetone solutions. He reports none on pure β-hydroxybutyric acid, although in diabetic

ketosis that substance constitutes about four-fifths of the "total acetone bodies."

We have repeated Smith's distillation procedure with mercury precipitates obtained in determinations of acetone plus acetoacetic acid and of total acetone bodies in a number of diabetic urines, and have controlled the results by performing similar determinations with solutions containing known amounts of both acetone and β-hydroxybutyric acid. Proper correction was made, as described in our original paper, for the mercury precipitate yielded by non-volatile substances in the urine filtrates. The results fail to confirm Smith's criticisms; in fact the data constitute additional proof that the writer's method is extremely specific for acetone, acetoacetic acid, and β-hydroxybutyric acid among the constituents of human urine.

When pure acetone solutions were precipitated and the acetone from the redissolved precipitate was distilled and titrated, the yield per gram of precipitate depended somewhat on the amount of precipitate. When the latter was in the neighborhood of 500 mg., (representing 25 mg. of acetone) distillation yielded 97 to 98 per cent of the expected 5 per cent of the precipitate's weight of acetone. When the precipitate was a third as great the yield of titrated acetone from it was only 90 per cent of the expected (see Table I).

*Precipitates from the acetone plus acetoacetic acid of diabetic urines, when the prescribed correction was deducted for the small portion due to non-acetone substances, yielded, within 1 or 2 per cent, the same proportion of distilled acetone as precipitates of similar weight formed from solutions of pure acetone* (compare Tables I and III).

Precipitates from pure hydroxybutyric acid yielded, as shown by Table II, about 10 per cent less distilled acetone than precipitates of the same weight from acetone solutions. It appears accordingly that when β-hydroxybutyric acid is boiled with sulfuric acid, chromate, and mercuric sulfate, under the conditions prescribed for determining β-hydroxybutyric acid or total acetone bodies, about 10 per cent of the precipitate formed is from an oxidation product or products other than acetone. As seen by comparison of Tables II and III, *precipitates from diabetic urines obtained in determination of the total acetone bodies yielded the*

*same proportion of distilled acetone as precipitates of the same weight from pure hydroxybutyric acid.* This fact adds to the evidence, quoted above from the writer's original paper (1917), that no significant part of the precipitate obtained in the determination of hydroxybutyric acid or total acetone bodies in ketone-containing urines originates from substances other than the acetone bodies sought.

The factors, by which hydroxybutyric acid or total acetone bodies are calculated from the weight of the precipitate obtained by the writer's method, were determined empirically by analyses of known solutions of pure acetone and $\beta$-hydroxybutyric acid (Van Slyke, 1917). These factors express the directly determined relation between the weight of precipitate and the amount of acetone or hydroxybutyric acid in the fluid analyzed, and involve no assumption concerning the amount of acetone recoverable from the precipitate. Hence the results in the present paper do not necessitate changing any of the factors used in calculating blood or urine acetone bodies content from the amount of precipitate obtained.

<div align="center">EXPERIMENTAL.</div>

The acetone used in the experiments recorded in Table I was prepared by redistilling Kahlbaum's preparation made from the sulfite compound. Stock solutions containing approximately 1 mg. per cc. were made by pipetting portions of the acetone into weighed measuring flasks partly filled with water, the acetone being measured by the increase in weight of the flask. These stock solutions when titrated for acetone with 0.1 N iodine solution and thiosulfate gave theoretical values.

The calcium-zinc salt of hydroxybutyric acid used for the experiments recorded in Table II was from the same lot of which the preparation (by the method of Shaffer and Marriott), analysis, and rotation are described in the original paper (Van Slyke, 1917).

The precipitates were all formed under conditions with regard to volume of solution, reagents, time of boiling, etc., which conform to those prescribed in the original paper (Van Slyke, 1917), for determination of acetone plus acetoacetic acid or of total acetone bodies.

The following procedure was used for distilling, and titrating by Messinger's method, the acetone from the mercury precipitates.

Each precipitate was collected, dried, and weighed in a Gooch crucible. It was then moistened with water, and the precipitate, together with the asbestos in the crucible, was transferred with the aid of a rod and wash water to a 500 cc. Kjeldahl flask. The volume of water in the flask was brought up to about 300 cc., and

TABLE I.

*Results with Solutions of Acetone.*

| Acetone present. | Weight of precipitate obtained. | 0.1 N iodine to titrate acetone distilled from precipitate. | Acetone calculated from weight of precipitate $= \frac{(b)}{20}$. | Acetone calculated from titration of distillate $= 0.967\ (c)$. | Ratio of distilled acetone to acetone calculated from precipitate $= \frac{(e)}{(d)}$. |
|---|---|---|---|---|---|
| (a) | (b) | (c) | (d) | (e) | |
| *mg.* | *mg.* | *cc.* | *mg.* | *mg.* | |
| 2 | 37.3 | 1.59 | 1.86 | 1.54 | 0.830 |
|   | 38.1 | 1.61 | 1.91 | 1.56 | 0.820 |
| 4 | 76.9 | 3.49 | 3.85 | 3.37 | 0.876 |
|   | 79.8 | 3.57 | 3.99 | 3.45 | 0.865 |
| 8 | 156.3 | 7.29 | 7.82 | 7.05 | 0.902 |
|   | 161.5 | 7.53 | 8.07 | 7.28 | 0.902 |
| 10.63 | 214.4 | 10.13 | 10.72 | 9.80 | 0.915 |
|   | 207.8 | 9.93 | 10.37 | 9.60 | 0.926 |
|   | 207.0 | 9.93 | 10.35 | 9.60 | 0.927 |
| 21.26 | 421.3 | 20.82 | 21.06 | 20.13 | 0.956 |
|   | 409.0 | 20.64 | 20.45 | 19.96 | 0.976 |
|   | 422.0 | 21.22 | 21.10 | 20.52 | 0.972 |

15 cc. of concentrated hydrochloric acid were added, dissolving the precipitate. The flask was immediately closed with a stopper bearing a Kjeldahl trap which was connected with a glass condenser. The outlet tube at the bottom of the condenser dipped below the surface of 150 cc. of water in a 500 cc. flask, which was cooled in an ice bath. The distillation was continued for 25 to 30 minutes. Longer distillation did not increase the yield.

To the distillate 10 cc. of 40 per cent KOH were added, and an excess of 0.1 N iodine solution. The flask was covered with a watch-glass and permitted to stand at room temperature for 20 minutes. Then sufficient concentrated hydrochloric acid (5.5 cc.) was added to neutralize the KOH and provide an excess of 0.2 cc. of acid. The solution was gently shaken to mix the reagents and the excess iodine was titrated with thiosulfate until the brown color had nearly disappeared. Then a few cc. of 5 per cent starch

TABLE II.

*Results with Solutions of β-Hydroxybutric Acid.*

| Hydroxybutyric acid present. | | Weight of precipitate. | 0.1 N iodine to titrate acetone distilled from precipitate. | Acetone calculated from weight of precipitate $= \frac{(b)}{20}$. | Acetone calculated from titration of distillate = 0.967 (c). | Ratio of distilled acetone to acetone calculated from precipitate $= \frac{(e)}{(d)}$. |
|---|---|---|---|---|---|---|
| Calcium-zinc salt added. | Amounts of free acid equivalent to Ca-Zn salt. | | | | | |
| (a) | | (b) | (c) | (d) | (e) | |
| mg. | mg. | mg | cc. | mg. | mg. | |
| 12.0 | 19.6 | 83.2 | 3.50 | 4.16 | 3.38 | 0.813 |
| | | 80.8 | 3.39 | 4.04 | 3.28 | 0.812 |
| 24.0 | 19.3 | 161.4 | 6.93 | 8.07 | 6.70 | 0.830 |
| | | 161.6 | 6.95 | 8.08 | 6.72 | 0.832 |
| 56.0 | 45.0 | 379.8 | 16.44 | 18.99 | 15.90 | 0.837 |
| | | 381.0 | 16.64 | 19.05 | 16.12 | 0.842 |
| | | 382.2 | 16.85 | 19.11 | 16.30 | 0.853 |
| 112.0 | 90.0 | 765.6 | 32.59 | 38.28 | 32.59 | 0.850 |
| | | 772.2 | 32.77 | 38.61 | 32.77 | 0.848 |
| | | 779.2 | 32.80 | 38.96 | 32.80 | 0.842 |

solution were added and the titration was continued until the blue color disappeared.

Blank determinations were performed in which asbestos mats from control Gooch crucibles were washed into Kjeldahl flasks, acidified, distilled, and the distillates were titrated in the same manner. The iodine used in the blank, about 0.4 cc. of 0.1 N solution, was subtracted from that used in the titration of the acetone distillates.

TABLE III.

*Results with Ketone-Containing Urines.*

| Urine No. | Determination | Precipitate (a) | Blank precipitate due to non-acetone substances (b) | Precipitate corrected for blank = (a) − (b). (c) | 0.1 N iodine to titrate acetone distilled from precipitate (d) | Acetone calculated from corrected weight of precipitate. (e) | Acetone calculated from titration of distillate = 0.967 (d). (f) | Ratio of distilled acetone calculated from precipitate = $\frac{(f)}{(e)}$ tate |
|---|---|---|---|---|---|---|---|---|
| | | $mg$ | $mg$ | $mg$ | $cc$ | $mg$. | $mg$. | |
| 1 | Acetone + aceto-acetic acid. | 123.6 | 4.6 | 119.0 | 5.24 | 5.95 | 5.07 | 0.853 |
| | | 123.4 | 4.6 | 119.0 | 5.19 | 5.95 | 5.02 | 0.843 |
| | Total acetone bodies | 40.34 | 4.6 | 398.8 | 17.28 | 19.94 | 16.70 | 0.837 |
| | | 40.46 | 4.6 | 400.0 | 17.25 | 20.00 | 16.69 | 0.834 |
| 2 | Acetone + aceto-acetic acid. | 164.2 | 2.6 | 161.6 | 7.50 | 8.08 | 7.25 | 0.897 |
| | | 166.0 | 2.6 | 163.4 | 7.67 | 8.17 | 7.42 | 0.908 |
| | Total acetone bodies. | 564.2 | 2.6 | 561.6 | 24.04 | 28.08 | 23.25 | 0.828 |
| | | 565.6 | 2.6 | 563.0 | 24.38 | 28.15 | 23.60 | 0.838 |
| 3 | Acetone + aceto-acetic acid. | 158.4 | 4.0 | 154.0 | 7.21 | 6.97 | 7.70 | 0.905 |
| | | 160.8 | 4.0 | 156.8 | 7.29 | 7.05 | 7.84 | 0.897 |
| | Total acetone bodies. | 428.2 | 4.0 | 424.2 | 19.12 | 18.5 | 21.21 | 0.872 |
| 4 | Acetone + aceto-acetic acid. | 122.0 | 3.2 | 118.8 | 5.36 | 5.94 | 5.18 | 0.872 |
| | | 121.2 | 3.2 | 118.0 | 5.34 | 5.90 | 5.16 | 0.874 |
| | Total acetone bodies. | 405.6 | 3.2 | 402.4 | 17.90 | 20.12 | 17.32 | 0.861 |
| | | 408.6 | 3.2 | 405.4 | 18.05 | 20.27 | 17.41 | 0.860 |
| 5 | Acetone + aceto-acetic acid. | 51.8 | 1.2 | 50.6 | 2.17 | 2.53 | 2.10 | 0.830 |
| | | 51.2 | 1.2 | 50.0 | 2.14 | 2.50 | 2.07 | 0.828 |
| | Total acetone bodies. | 151.5 | 1.2 | 150.3 | 6.44 | 7.52 | 6.22 | 0.827 |
| | | 150.2 | 1.2 | 149.0 | 6.40 | 7.49 | 6.19 | 0.826 |
| 6 | Acetone + aceto-acetic acid. | 108.2 | 1.4 | 106.8 | 4.96 | 5.34 | 4.80 | 0.899 |
| | | 109.0 | 1.4 | 107.6 | 4.92 | 5.38 | 4.76 | 0.884 |
| | Total acetone bodies. | 322.4 | 1.4 | 321.0 | 14.28 | 16.05 | 13.80 | 0.860 |
| | | 317.4 | 1.4 | 316.0 | 14.03 | 15.80 | 13.56 | 0.859 |

Stock solutions of acetone distilled and titrated in this manner gave theoretical results.

### SUMMARY.

The basic mercuric salt precipitates, obtained by applying to diabetic urines the writer's methods for determination of acetone plus acetoacetic acid and of total acetone bodies (acetone plus acetoacetic acid plus $\beta$-hydroxybutyric acid) yield when dissolved and distilled, the same proportions of acetone as precipitates of equal weight obtained from solutions of pure acetone and $\beta$-hydroxybutyric acid respectively. This fact affords added evidence that no significant amount of the precipitate obtained from diabetic urines originates from substances other than the acetone bodies. Smith's contrary conclusion is attributable to his failure to carry out control analyses with pure hydroxybutyric acid.

The analyses reported above were performed by Mr. John Plazin.

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# MANOMETRIC DETERMINATION OF PRIMARY AMINO NITROGEN AND ITS APPLICATION TO BLOOD ANALYSIS.

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The writer has previously published (Van Slyke, 1910, 1911) a procedure for determination of aliphatic amino nitrogen by measurement of the $N_2$ gas evolved by the reaction with nitrous acid.

$$RNH_2 + HNO_2 = ROH + 2 H_2O + N_2$$

A special apparatus was devised, which was later improved (1912) and developed for micro analyses (1913–14, 1915).

The more generally useful Van Slyke-Neill manometric apparatus, however, gives equally rapid and accurate amino nitrogen determinations, and makes possible micro analyses with smaller amounts of material than even the micro type of the former apparatus. This advantage in favor of the manometric apparatus is due partly to the greater accuracy with which it permits measurement of small gas amounts, partly to its permitting one to use larger volumes of amine solution. In consequence, whereas the micro form of the previous apparatus permitted measurement of amino nitrogen concentration in 2 cc. of solution to ± 0.001 mg. per cc., the manometric apparatus permits measurement in a 5 cc. sample to ± 0.0001 mg. per cc., this amount causing a change in the manometer reading of 1.3 mm. With the manometric apparatus one can accordingly perform a blood amino nitrogen determination directly on 5 cc. of the Folin-Wu tungstic acid blood filtrate, without the concentration to smaller volume which was formerly a necessary preliminary to analysis.

For discussion of the principle of the reaction and the manner

in which varying types of aliphatic amines react with nitrous acid under the conditions employed, the reader is referred to the original paper (Van Slyke, 1911). The most significant facts are that the $NH_2$ groups in the $\alpha$-amino acids react quantitatively in 3 to 4 minutes at room temperature; while $NH_2$ groups in other types of substances react much more slowly. Of ammonia about 25 per cent reacts in the time required for complete reaction of $\alpha$-amino acids (Van Slyke, 1912), and of urea only 6 to 7 per cent (Levene and Van Slyke, 1912).

The reaction is carried out by mixing three solutions; *viz.*, of sodium nitrite, acetic acid, and amine. In the original special apparatus (Van Slyke, 1910, 1911, 1912) the order of addition was obligatory: the nitrite and acetic acid had to be added first, and shaken until the NO gas evolved by spontaneous decomposition of $HNO_2$ had washed all the air out of the reaction chamber, before the amine solution was added. In the manometric apparatus any two of the reagents, AcOH and $NaNO_2$, $NaNO_2$ and $RNH_2$ (in neutral or alkaline solution), or AcOH and $RNH_2$, may be mixed and freed of air in the extraction chamber, and the third reagent then added. The last of the above three orders proved in general to be preferable. The amine solution and acetic acid are mixed and freed of air in the chamber, and the $NaNO_2$ is then added in saturated solution. The saturated nitrite solution need not be freed of dissolved air before it is used. Because of its high salt content (60 grams per 100 cc.), this solution when saturated with air at room temperature dissolves only 0.2 volume per cent of the atmospheric gases (one-tenth as much as water), of which one-third is $O_2$ and disappears by combination with NO during the reaction. The amount of dissolved atmospheric $N_2$ carried into the apparatus by the 2 cc. of nitrite solution exerts only 4 mm. of pressure when the gas is measured at 0.5 cc. volume, 1 mm. when at 2 cc. The corrections for these small amounts of dissolved air are too small to vary measurably with room temperature or barometric pressure, and are automatically included in the blank analysis on the reagents.

The entire procedure requires 12 to 15 minutes. The maximum amount of amino nitrogen that can be determined in a sample is about 0.6 mg., which at 2 cc. volume yields nitrogen gas giving a little over 400 mm. of pressure. The minimum amount meas-

urable in micro determinations is about 0.0004 mg., which yields nitrogen **gas** giving 1 mm. of pressure at 0.5 cc. volume. Since samples of 5 cc., and, if desired, greater volume can be analyzed, a concentration of 0.01 mg. of amino nitrogen per cc. suffices for an analysis capable of 1 per cent accuracy.

### DESCRIPTION OF METHOD.

### *Reagents.*

*Sodium Nitrite Solution.*—800 grams of $NaNO_2$ dissolved with the aid of warming in 1 liter of water.

*Glacial Acetic Acid.*

*Alkaline Permanganate.*—50 grams of $KMnO_4$ are shaken with 1 liter of 10 per cent NaOH solution until the latter is saturated with the permanganate.

*Caprylic Alcohol.*—This is used when necessary to prevent foaming of viscous solutions.

### *Procedure.*

The analysis consists of the following steps.

1. The amine solution and acetic acid are freed of air in the apparatus.

2. Sodium nitrite solution is added and the resultant nitrous acid is permitted to react for the necessary time, 3 to 4 minutes in the case of $\alpha$-amino acids at room temperatures of 25–20°.

3. The mixture of $N_2$ and NO (the latter formed by spontaneous decomposition of $HNO_2$) is transferred to a Hempel pipette of the type described by Van Slyke and Hiller (1928), where the NO is absorbed by permanganate.[1]

4. The chamber of the Van Slyke-Neill apparatus is washed

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[1] It is possible by using the Harington-Van Slyke (1924) type of extraction chamber to avoid the use of the Hempel absorption pipette. The nitrous acid solution is drawn off and ejected through the trap at the bottom of the Harington-Van Slyke chamber, which is then washed with absolutely air-free 20 per cent NaOH solution, followed by air-free permanganate, over which the residual $N_2$ is finally measured. However, in practice this procedure has proved less convenient and certain than that described in the text. Introduction of the slightest trace of air with the alkali or permanganate suffices to invalidate a micro amino determination. And the permanganate each time fouls the mercury, although the latter is instantly cleaned by contact with the next nitrous acid solution.

free of nitrous acid, and the purified $N_2$ gas is returned from the pipette.

5. The amount of $N_2$ gas is measured by the pressure it exerts at either 0.5 or 2.0 cc. volume.

The details of the successive steps follow.

*1. Removal of Air from Mixed Solution of Amine and Acetic Acid.*—The sample of amine solution may vary from 1 to 8 cc. in volume. 5 cc. is usually a convenient size. The amine solution is run into the chamber of the Van Slyke-Neill apparatus, followed by 1 cc. of glacial acetic acid. The most convenient way to add the amine solution is to run it directly into the chamber from a rubber-tipped, stop-cock pipette, as shown in Fig. 3 of the paper on the portable manometric apparatus (Van Slyke, 1927). However, one may run the solution from an ordinary pipette into the cup of the apparatus and wash into the chamber with small amounts of water, or with the 1 cc. of acetic acid divided into three or four portions. The total volume of fluid added should be known, because the time required for the subsequent reaction is proportional to the dilution of the reagents. If protein or other content of the amine solution makes the latter likely to form troublesome foam, a drop of caprylic alcohol is added with the acetic acid. The amine solution and acetic acid being in the chamber, the cock of the latter is sealed with a drop of mercury, and the chamber is evacuated and shaken for 2 minutes at the usual tempo of 250 to 300 times per minute.

The air extracted from the solution is then ejected from the chamber as follows: The leveling bulb is placed in its uppermost ring, slightly above the chamber. The cock admitting mercury from the leveling bulb into the chamber is opened, so that the extracted air is compressed into a bubble at the top of the chamber. The cock from the leveling bulb is now closed, and the cock at the top of the chamber (see Fig. 1) is opened. Part of the air escapes by its own pressure. The rest is expelled by admitting mercury slowly from the leveling bulb, until the solution in the chamber has risen just high enough to expel the gas and fill the capillary above Cock *b*.

*2. Decomposition of Amino Groups.*—2 cc. of the nitrite solution are measured into the chamber. Evolution of $N_2$ and NO begins at once. The cock is sealed with a drop of mercury, and the
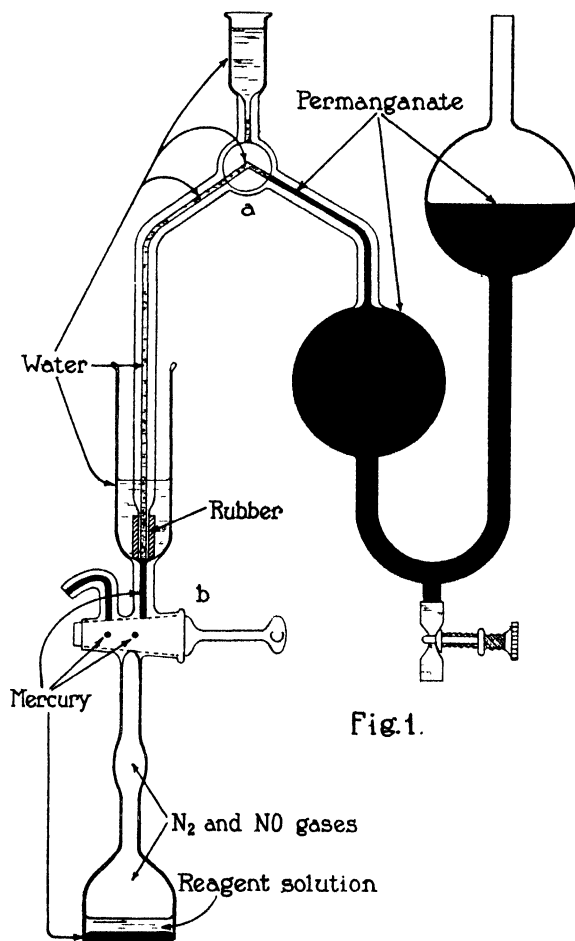
FIG. 1. Apparatus arranged for transfer of $N_2 + NO$ gas mixture to Hempel pipette by turning Cock $b$.

chamber is evacuated until the mercury in it has fallen to a level 1 or 2 cm. above the 50 cc. mark. The reaction mixture is permitted to stand in this position until within 1 minute of the end of
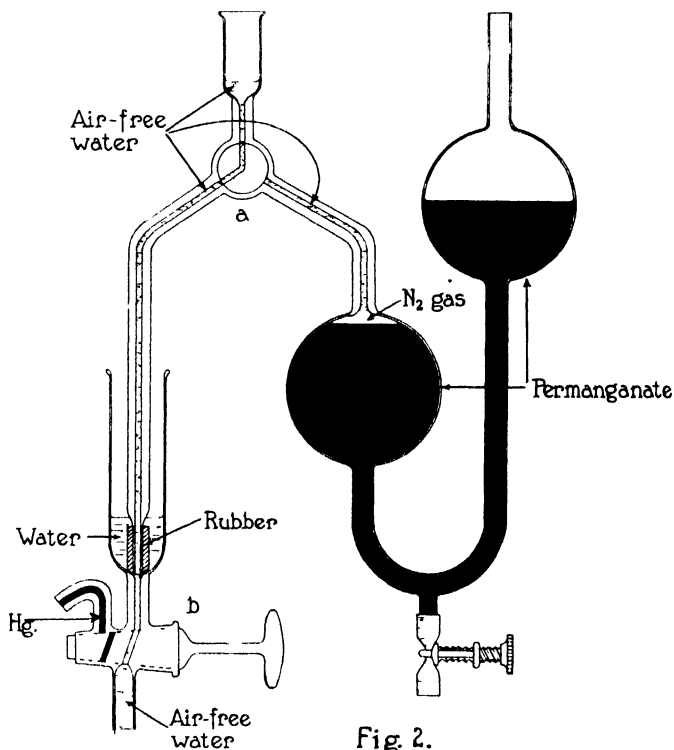


Fig. 2.

FIG. 2. After absorption of NO by permanganate in Hempel pipette, and replacement of reagents by air-free water in extraction chamber, the Hempel pipette is placed again in connection with the chamber, and water from the chamber is forced up through the capillary into the cup of the pipette.

the reaction time, given below. During the last minute the mixture is shaken to complete the evolution of the $N_2$ formed. The relatively large amount of NO gas evolved with the $N_2$ by spon-

taneous decomposition of the nitrous acid tends to press the mercury meniscus down into the tube below the chamber. To prevent this, one admits mercury from the leveling bulb once or twice during the shaking, so that the mercury meniscus in the chamber remains within a centimeter of the 50 cc. mark.
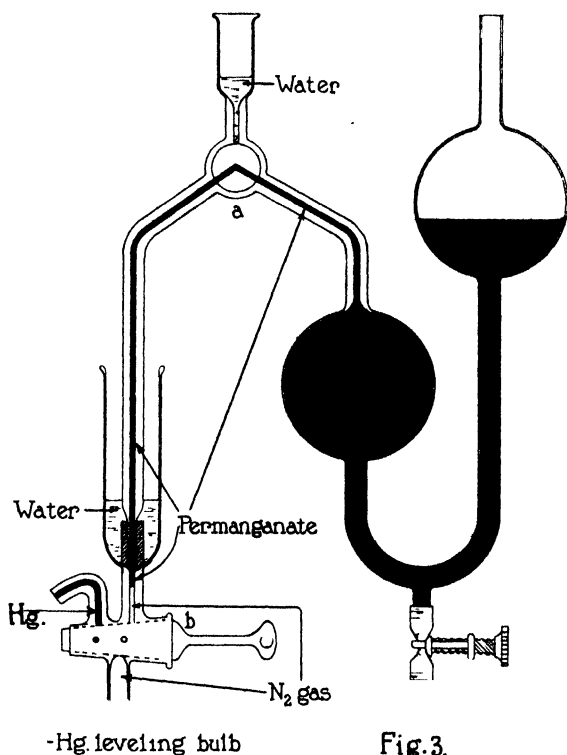


FIG. 3. Mercury-leveling bulb has been lowered and $N_2$ gas drawn back to chamber, followed by permanganate solution as far as point indicated.

The time required for complete decomposition of the alpha $NH_2$ groups of amino acids, measured from the moment when the nitrite is run into the chamber till the end of the minute of shaking, varies with the temperature, and at a given temperature it is proportional to the volume to which the reagents are diluted. When the amine solution plus the water added with it is 5 cc., so

that the total volume of solution in the chamber is 8 cc., the time required for quantitative reaction of $\alpha$-amino acids is 3 minutes at 25°, 4 minutes at 20°, and 6 minutes at 15°, as indicated by Fig. 4. If the volume of the mixed solutions is greater or less than 8 cc., the reaction time is proportionally increased or diminished.

*3. Transfer of NO $+$ N₂ Gas Mixture to Permanganate Pipette, and Absorption of NO.*—After the reaction between amine and nitrous acid is completed, the mercury leveling bulb of the Van Slyke-Neill apparatus is raised to the level indicated in Fig. 1, and the cock (not shown in the figure) connecting the leveling bulb with the gas chamber is opened. The mercury rises in the chamber, and the gases in it collect at the top under positive pressure, as shown in Fig. 1. 2 or 3 cc. of water are placed in the cup above the chamber, and the Hempel pipette, with its capillaries filled with water, is pressed firmly into the position shown in Fig. 1. Cock $a$ is turned into position as shown in Fig. 1. Then by opening Cock $b$ the gases are forced over into the pipette. When the nitrous acid solution, following the gases, has travelled up the pipette capillary nearly as far as Cock $a$, both Cocks $b$ and $a$ are closed. It is preferable not to let any of the nitrous acid solution pass over into the permanganate, because it exhausts the latter unnecessarily.

With Cock $a$ in a position intermediate between those shown in Figs. 1 and 2, the Hempel pipette is disconnected from the extraction chamber. The gas in the capillary between Cock $a$ and the permanganate bulb is forced down into the latter by water from the cup. The remainder of the water in the cup is driven out through the left hand capillary, to wash the nitrous acid solution out of it.

The disconnected Hempel pipette is gently shaken horizontally by hand to absorb the NO gas. The time required is 20 to 40 seconds, depending on whether the amount of N₂ approximates the minimum or maximum determinable by the method. The pipette is then set aside. (It may be conveniently suspended from a hook at the right of Cock $a$.)

*4. Return of Purified N₂ Gas to Manometric Apparatus.*—Before the N₂ is returned to the gas chamber, the nitrous acid solution is ejected from the latter, which is then washed twice by the following technique. The mercury leveling bulb is placed in its ring

at the low level, where it evacuates the chamber. As the mercury in the latter falls, 10 or 15 cc. of water, but no air, are admitted to the chamber from the cup at its top. The bulb is then raised, and the water is ejected. 30 seconds suffice for each washing.[2] After the second washing 10 cc. of water, measured in two portions from the cup, are admitted into the chamber, which is evacuated and shaken for 1 minute to remove the greater part of the air from the water. The extracted air is ejected, and 1 cc. of the water is forced up into the cup above the chamber.

The $N_2$ gas from the Hempel pipette is now returned to the chamber by connecting as shown in Fig. 3. The capillary between Cocks $a$ and $b$ is filled with water from the chamber, and a little water is forced up into the cup above Cock $a$. The mercury leveling bulb is now lowered to its middle position, level with the bottom of the extraction chamber and the $N_2$ is admitted from the pipette to the chamber. The flow of gas to the chamber can be regulated either by Cock $b$ or by the cock (not shown in the figures) which connects the chamber to the mercury bulb. The writer prefers the mercury cock because of the nicety with which the flow of the mercury, and thereby that of the other fluids, can be regulated. The flow is stopped and Cock $b$ is closed when the column of permanganate has reached the position shown in Fig. 3, in the capillary above Cock $b$. It is preferable to get as little permanganate into the chamber below as possible, in order to have a clear water meniscus there for reading.

After removing the Hempel pipette the cup of the gas chamber is washed with water to remove permanganate that may have escaped into it, and about 1 cc. of mercury is run underneath water into the cup. As much of this mercury is run into the chamber as may be necessary to clear of permanganate the bore of Cock $b$ and the constricted top of the chamber below the cock.

*5. Measuring the Nitrogen Gas.*—The level of the water in the chamber is lowered until the water meniscus is at either the 0.5 cc.

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[2] In case large amounts of protein are present in the solution analyzed, deaminized protein is precipitated and floccules of it are likely to adhere to the walls of the chamber when the nitrous acid is ejected. Such particles are dissolved by running in a few cc. of 5 or 10 per cent NaOH solution, and raising and lowering the mercury and the alkali solution in the chamber. The latter is then washed twice with water, as above described.

or the 2.0 cc. mark, according to the amount of gas present. If the latter at the 2.0 cc. mark exerts less than 100 mm. pressure it is preferable to use the 0.5 cc. mark, employing a reading glass to locate the meniscus exactly on the line. The manometer reading, $p_1$, is taken. The gas is ejected from the chamber by the technique described for the first step of the analysis, and the manometer reading $p_0$ is taken with the water meniscus in the gas-free chamber at the same level used for the $p_1$ reading.

*Blank Analysis of Reagents.*—A control analysis is carried out in the same manner, except that an equal volume of water replaces the amine solution. The difference $p_1 - p_0$ obtained in the control is the $c$ correction used in the calculation below. For some days or weeks after the sodium nitrite solution has been prepared the $c$ correction appears to diminish, and finally to become constant. It is convenient to set aside a considerable amount of nitrite solution and acetic acid on which the $c$ correction has been determined, in order to avoid necessity of frequent redetermination on new solutions. With the Merck's "Reagent" nitrite used by us the $c$ correction has been 20 to 30 mm. measured with the gas at 0.5 cc. volume, and one-fourth as much at 2.0 cc. volume. When there is sufficient amino nitrogen to give over 100 mm. pressure at 2 cc. volume, variations of the $c$ correction with ordinary variation in room temperature may be neglected. For micro analyses, however, with gas measurements at the 0.5 cc. mark the $c$ correction must be determined at a temperature near that of the analysis.

*Calculation.*—The pressure of $N_2$ gas from the amine analyzed is calculated as

$$P_{N_2} = p_1 - p_0 - c$$

whence the weight of amino nitrogen in the sample is calculated as

$$\text{Mg. amino N} = P_{N_2} \times \text{factor}$$

The values of the factor are found in Table I.

These values are calculated from Equation 5 of Van Slyke and Neill (1924); *viz.*,

$$\text{mm gas} = P \times \frac{1}{17{,}024\ (1 + 0.00384\ t)} \left(1 + \frac{S}{A - S}\,\alpha'\right)$$

where $P$ is the observed mm. pressure of gas at $a$ cc. volume, $S$ the cc. of solution present in the extraction chamber of $A$ cc. capacity, and $\alpha'$ is the Ostwald solubility coefficient of the gas in the solution. The millimols obtained by the equation are multi-

TABLE I.

*Factors for Calculation of Amino Nitrogen.*

| Temperature. | Factors by which mm. $P_{N_2}$ are multiplied to give mg. amino N in sample analyzed. | | Factors by which mm. $P_{N_2}$ are multiplied to give mg. amino N per 100 cc. blood when filtrate sample is equivalent to 0.5 cc. blood. |
|---|---|---|---|
| | $a = 0.5$ cc. | $a = 2.0$ cc. | $a = 0.5$ cc. |
| °C. | | | |
| 15 | 0 000390 | 0.001561 | 0.0780 |
| 16 | 389 | 55 | 777 |
| 17 | 387 | 49 | 774 |
| 18 | 386 | 44 | 772 |
| 19 | 385 | 38 | 769 |
| 20 | 383 | 33 | 766 |
| 21 | 382 | 27 | 763 |
| 22 | 380 | 22 | 761 |
| 23 | 379 | 16 | 758 |
| 24 | 378 | 11 | 756 |
| 25 | 376 | 06 | 753 |
| 26 | 375 | 00 | 750 |
| 27 | 374 | 0.001495 | 748 |
| 28 | 372 | 90 | 745 |
| 29 | 371 | 85 | 743 |
| 30 | 370 | 80 | 740 |
| 31 | 368 | 74 | 737 |
| 32 | 367 | 69 | 734 |
| 33 | 366 | 64 | 732 |
| 34 | 365 | 59 | 730 |

plied by $\dfrac{28.02}{14.01}$ to obtain mg. of amino nitrogen, since of each millimol (28.02 mg.) of $N_2$ yielded by the reaction with nitrous acid only half comes from the amine. The value in the equation for the reabsorption factor, $i$, in the case of $N_2$ is 1,

because no measurable reabsorption of $N_2$ occurs under the conditions of the analysis.

The factor $(1 + \dfrac{S}{A - S} \, \alpha')$, correcting for the solubility of $N_2$, affects in this analysis the results by only about 1 part in 700, which is within the limit of error. We have, however, included it in the calculations. It proved impracticable to determine the solubility of $N_2$ directly in the reaction mixture, because of the continuous effervescence of NO gas. The value for $\alpha_{N_2}$ in water containing the concentration of sodium nitrite present in the 8 cc. of reacting solution, however, we have found to be 50 per cent of $\alpha_{N_2}$ in pure water. Acetic acid in the concentration present does not alter the solubility of $N_2$ from that in pure water to an extent significant in these calculations. The acid dissociation constant of $HNO_2$ is about 25-fold that of AcOH (see Landolt-Börnstein's "Talbellen'). Hence, with equimolar proportions of $NaNO_2$, and AcOH present, about 0.96 of the nitrite keeps the form of the sodium salt, and about 0.96 of the AcOH the form of free acetic acid. One appears justified in assuming that the effects on $N_2$ solubility in the mixed solution are approximately the added effects of the $NaNO_2$ and AcOH.

Equation 5 of Van Slyke and Neill becomes therefore, for our present case,

$$\text{Mg. amino N} = P_{N_2} \times \frac{14.01 \, a}{17{,}024 \, (1 + 0.00384 \, t)} \left( 1 + \frac{8}{42} \times \frac{\alpha'_{N_2}}{2} \right)$$

$$= P_{N_2} \times \frac{0.0008230 \, a}{1 + 0.000384 \, t} \, (1 + 0.095 \, \alpha'_{N_2})$$

$$= P_{N_2} \times \text{factor}$$

where $\alpha'_{N_2}$ indicates the solubility of $N_2$ in pure water, as given in Table I of Van Slyke and Neill.

In the case of blood analyses, the 5 cc. of filtrate represent 0.5 cc. of blood. Consequently $P_{N_2}$ is multiplied by 200 times the above factor in order to obtain mg. of amino nitrogen per 100 cc. of blood.

When less than 5 cc. of water are added with the amine solution, so that total solution volume, $S$, is less than 8 cc., the solubility

correction will be even less than that calculated by the factor $(1 + 0.095\ \alpha'_{N_2})$. Since the difference, however, would affect results by less than 1 part in 1000, the same factors, in Table I of this paper, may be used when the volume of the reaction mixture varies by 3 cc. on either side of the 8 cc. assumed in the calculation.

### Shortened Procedure for Series of Analyses.

When a number of determinations are performed in succession, it is convenient to change the procedure to the following.

The above described $p_0$ reading is omitted in the amine analysis, the latter being concluded after $p_1$ is noted. The $p_1$ reading of the blank analysis is taken as $p_0$ for the amine analysis. Then

$$P_{N_2} = p_1 - p_0$$

The $p_0$ thus determined in the blank analysis includes the correction for impurities in the reagents, so that there is no $c$ correction to subtract. The advantage of this procedure is that it obviates one reading with each analysis. The disadvantage is that the $p_0$ varies with the temperature, due chiefly to effect on vapor tension in the chamber. If the temperature rises between the time of the blank analysis and the subsequent amine analysis, 1.5 mm. may be added to $p_0$ for each degree rise, subtracted for each degree fall. If the temperature change exceeds $2°$, however, it is well to redetermine $p_0$.

### Use of Modified Hempel Pipette.

One's ability to perform easily and without the loss of 0.001 cc. of gas the transfers to and from the modified Hempel pipette depends upon proper construction of the latter. The rubber tip, made from soft tubing of 7 mm. outer diameter and 2 mm. bore, should project a hair's breadth below the glass tip of the pipette, and should be so shaped that it fits snugly into the bottom of the cup of the Van Slyke-Neill chamber. The bore of the capillaries of the pipette should be between 0.9 and 1.1 mm. In particular the 3-way cock must be accurately ground, and the bore through the stopper must maintain its diameter sharply to the surface of the stopper. If the bore is widened out funnel-like at the ends, traps are formed in which small gas bubbles are likely to be held

back, sufficient to affect significantly the results of a micro analysis, such as is performed on blood filtrates. The 120° angle in the bore of the cock must be exact, so that in all of its three positions the cock will unite the connected capillaries into smoothly continuous tubes.

It is desirable for each day's analyses to fill the pipette with fresh permanganate solution saturated with air at room temperature. One makes certain that the solution is at room temperature, and then whirls 75 cc. of it about the walls of an open 1 liter flask for 1 or 2 minutes, in order to bring it into equilibrium with air at atmospheric pressure before the solution is put into the pipette.

During the course of a series of analyses a film of manganese dioxide forms on the wall of the pipette near the capillary outlet. When the permanganate is renewed the film is as a rule readily detached by shaking water in the pipette. Film which becomes adherent is removed by washing with a saturated solution of oxalic acid in normal sulfuric acid.

Theoretically one would anticipate some error from contact of the pure $N_2$ gas, left after absorption of NO, with the permanganate solution in the Hempel pipette. The permanganate solution is saturated, not with pure $N_2$, but with the $N_2$-$O_2$ mixture of the atmosphere. The water of the permanganate solution must give off some $O_2$ to the nitrogen bubble, and absorb some of the $N_2$. In control analyses we have found, however, that the amount of such exchange which occurs decreases the volume of gas, returned as $N_2$ to the gas chamber, by only about 0.0012 cc., sufficient to lower the $p_1$ value 2 mm. when measured at 0.5 cc. volume, or 0.5 mm. when measured at 2.0 cc. Error even of this small extent is, however, avoided by using a $c$ correction determined by the same technique, with the same slight loss of $N_2$.

*Determination of Amino Acid Nitrogen in Blood.*

Of the nitrogenous constituents of protein-free blood filtrates not only the amino acids, but also the urea reacts measurably with nitrous acid under the conditions of the analysis. In the time used for complete reaction of the $\alpha$-amino acids about 7 per cent of urea nitrogen is decomposed. In human blood without pathological urea retention the urea nitrogen is ordinarily about twice,

at most three times, the amino acid nitrogen content. Under these conditions the amino nitrogen can be determined without preliminary removal of the urea, a correction of 0.07 of the urea nitrogen being subtracted from the total nitrogen obtained by the nitrous acid reaction.

When, however, there is gross urea retention, with blood urea nitrogen above 50 mg. per 100 cc., it is desirable for exact results to remove the urea. The removal is easily accomplished with urease, the resultant ammonia being boiled off before the amino nitrogen is determined.

Accordingly two procedures are described for blood analysis.

*Method A. For Blood of Normal or Moderately Increased Urea Content.*

5 cc. of blood filtrate, prepared by the tungstic acid method of Folin and Wu (1919), and representing 0.5 cc. of blood, are pipetted into the chamber of the gas apparatus and analyzed as above described. The only difference in detail is that in the present analysis the time of reaction, measured *from the moment the sodium nitrite solution is run into the chamber to the end of the 1 minute shaking*, must be regulated with regard to the temperature somewhat more carefully than is ordinarily necessary, in order that the proportion of urea decomposed shall approximate the constant value of 0.07 allowed for. The reaction periods used for different room temperatures are indicated on the scale of Fig. 4. With a stop-watch or interval timer one can readily control the reaction time within 10 seconds.

The urea content of the blood is determined independently.

From the amino nitrogen content of the blood calculated by Table I, 0.07 of the urea nitrogen content is subtracted, to correct for $N_2$ evolved from that proportion of the urea.

Fig. 4 is constructed in accordance with the assumption that the speed of reaction between urea and nitrous acid obeys the usual temperature rule of time reactions at room temperature, which double in speed with each 10° temperature increase in accordance with the exponential formula

$$\frac{\theta_2}{\theta_1} = 10^{0.03 \, (t_1 - t_2)}$$

where $\theta_1$ and $\theta_2$ are the periods required at temperatures $t_1$ and $t_2$ for the reaction to proceed to a given point.   That, for the reaction between urea and nitrous acid under the conditions of the analysis, the above temperature rule is followed with a sufficiently close degree of approximation is indicated by the results of Levene
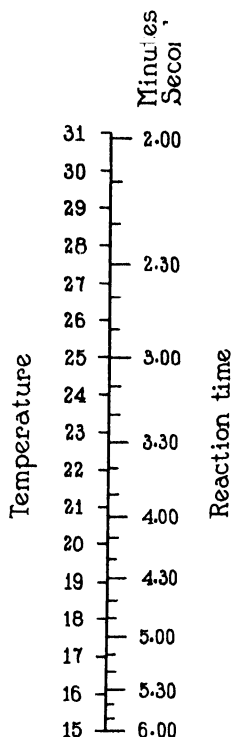


Fig. 4. Scale indicating reaction period required for complete decomposition of $\alpha$-amino acids, and 0.07 decomposition of urea, when total volume of reacting solution is 8 cc.

and Van Slyke (1912), and confirmed by additional analyses with the present technique.

*Method B.   For Blood of Either Normal or High Urea Content.*

In this procedure the urea is destroyed with urease.   It is desirable to use a relatively small proportion of urease in order to keep down the correction for amino acids in the urease preparation.

The blood sample, 1 to 5 cc., is placed in a flask calibrated to hold 10-fold the volume of the sample. For each cc. of blood 1 cc. of a 0.6 per cent $KH_2PO_4$ solution and 0.02 cc. of a 10 per cent aqueous solution of Squibb's jack bean urease are added. The mixture is permitted to stand an hour at a room temperature of 20° or over.[3]

The proteins are precipitated with 10 per cent sodium tungstate and 2/3 N sulfuric acid as described by Folin and Wu (1919), with the modification that *in uremic blood enough extra 2/3 N sulfuric acid is added to neutralize the ammonia* formed from the urea. If the blood urea nitrogen is 100 mg. per 100 cc., 0.1 cc. of 2/3 N sulfuric acid is added per cc. of blood in addition to the 1.0 cc. ordinarily used. Otherwise some protein may come through into the filtrate. The precipitated blood mixture is brought to 10-fold the volume of the blood and filtered.

To boil off the ammonia formed from the urea, 5, 10, 15, or 20 cc. of filtrate are measured into a Pyrex Erlenmeyer flask of 25 to 150 cc. capacity and a few drops of magnesium hydroxide suspension are added, sufficient to make the entire solution turn white. The mixture is boiled for 5 or 10 minutes in the open flask until the volume has been reduced about one-half. Glacial acetic acid is then added, a drop at a time, until the solution turns acid and the magnesium hydroxide dissolves.[4]

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[3] The enzyme is prepared from jack beans by the acetone precipitation method of Van Slyke and Cullen (1914). With Squibb's urease of the quality at present provided, capable of decomposing per minute nearly 0.1 mg. of urea per mg. of dry urease, 0.02 cc. of 10 per cent urease solution per cc. of blood suffices, even in uremic cases. If an enzyme preparation is used which, when standardized as previously described (Van Slyke, 1927, pp. 714–716), proves to be much weaker, one must either use more or let it act longer. On the other hand half as much enzyme can be used if the digestion time is doubled, or the temperature raised to 30°.

[4] Boiling with magnesium hydroxide is used to remove ammonia because Osborne, Leavenworth, and Brautlecht (1908) found in analysis of hydrolyzed proteins that this treatment removed ammonia quantitatively without appreciably affecting the amino acids. Boiling with more powerful alkalies, even dilute alkali carbonates, splits off ammonia from some of the amino acids. We have used Phillips' milk of magnesia which by titration is equivalent to an alkali solution of 2.7 N concentration. Magnesium hydroxide suspension has over magnesium oxide the advantage that it forms an even suspension, and dissolves instantly as soon as an excess of acetic

If 10 cc. or more of filtrate have been used the contents of the Erlenmeyer flask are poured into a volumetric flask and the Erlenmeyer flask is washed with small portions of water until the sample has been brought back to its original 10, 15, or 20 cc. volume.   5 cc. portions are used for amino nitrogen determination.

If only 5 cc. of filtrate were boiled down, the Erlenmeyer flask is drained directly into the cup of the gas apparatus, and the volume noted, *e.g.*, 2.7 cc.   Then enough water to make this up to 5 cc is drawn into a graduated pipette, and is used in successive portions to wash the flask, whence the washings are poured into the cup of the gas apparatus, and from that passed into the chamber.

The *blank analysis* in this case includes some amino nitrogen from the urease (the latter is ordinarily free of ammonia), and is performed as follows: 1 cc. of the 10 per cent urease solution is placed in a 10 cc. flask.   0.5 cc. each of 10 per cent sodium tungstate and $2/3$ N sulfuric acid is added, the mixture is diluted to the mark, shaken, and, after a half hour's standing to flocculate the proteins, is filtered.   1 cc. of the filtrate is diluted to 50 cc. with water.

The blank analysis is performed with 5 cc. of the diluted filtrate instead of 5 cc. of water.[5]

*Calculation.* —The pressure of $N_2$ from amino acids in blood is calculated as

$$P_{N_2} = p_1 - p_0 - c$$

where $c$ is the $p_1 - p_0$ value determined in the above blank analysis.   With the urease we have used, the $c$ value, measured at 0.5 cc. gas volume, is increased several mm. by the non-protein urease constituents.   The effect could be diminished by using less

acid is added.                                                              .
The present method gives with normal blood values for amino nitrogen about 1 mg per 100 cc. higher than those obtained with the nitrous acid reaction by Bock (1917) presumably because Bock used strong alkali, KOH, to boil off the ammonia.

[5] The 50-fold dilution is used only when the amount of 10 per cent urease employed has been 0 02 cc. per cc. of blood.   In this case each cc. of blood-urease filtrate contains the non-protein constituents of 0 2 mg. of urease preparation, whereas the undiluted blank filtrate contains per cc. the non-

urease and longer or warmer digestion, or by using especially purified urease, but the correction is sufficiently small and constant to make such refinements appear unnecessary.

The amino nitrogen values by Method B are usually 0.1 to 0.3 mg. per cent lower than by Method A (*e.g.*, see Table IV). Apparently boiling with MgO decomposes a slight amount of some amino compound in the blood filtrate, although such treatment has been observed to split no ammonia from the amino acids yielded by protein hydrolysis.[5]

## EXPERIMENTAL.

### *Analyses of Leucine Solutions.*

Of leucine, prepared from hydrolyzed casein by the ester method and purified by precipitation as the lead salt, as described by Levene and Van Slyke (1909), 100.0 mg. were dissolved in 100 cc. of water. With 5 cc. portions the first five analyses of Table II were carried out as described above. A portion of solution was then diluted 10-fold, and used for the last five analyses of Table II. Since leucine contains 10.69 per cent of nitrogen, the theoretically calculated nitrogen contents of 5 cc. portions of the two solutions analyzed were 0.534 and 0.0534 mg., respectively. The fact that 99.5 instead of 100 per cent of these amounts was obtained may be due either to traces of impurity in the leucine preparation or to analytical error within the limit of accuracy of the determinations. Comparison of the results obtained with different reaction periods indicates that the time required for complete reaction, as indicated by maximum $N_2$ yield, is the same as in the former special amino apparatus, approximately 3 minutes at 25°, 4 minutes at 20°. That the time

protein constituents of 10.0 mg. of urease, or 50 times as much. In case more or less than 0.02 cc. of 10 per cent urease per cc. of blood is used, the extent to which the blank filtrate is to be diluted will vary accordingly, being represented by the denominator of the fraction of a cc. of urease solution used per cc. of blood.

The reason, in the blank analysis, for precipitating a relatively concentrated urease solution and diluting the filtrate instead of precipitating an already diluted urease solution, is that the latter would be so extremely dilute that the proteins could not be made to coagulate within a practicable time.

requirement would be nearly the same was to be expected from the fact that the concentrations of sodium nitrite and acetic acid in the reacting mixture are but slightly different from those used in the former apparatus. Under the conditions used in the latter, for each analysis, 1 cc. of glacial acetic acid and 0.96 gm. of $NaNO_2$ were diluted to a total volume of 7.5 cc. In the reaction

TABLE II.

*Analyses of 5 Cc. Samples of Leucine Solutions.*

| Concentration of leucine solution. | Duration of reaction. | Temperature. | $P_{N_1}$ | $N_2$ volume at $P_{N_2}$ reading. | Factor.* | Amino N found. | |
|---|---|---|---|---|---|---|---|
| per cent | min | °C. | mm. | cc. | | mg. | per cent of theoretical |
| 0.1000 | 1 | 25.0 | 322.0 | 2.004 | 0.001509 | 0.4972 | 91.0 |
| 0.1000 | 2 | 25.0 | 344.2 | 2.004 | 0.001509 | 0.5194 | 97.2 |
| 0.1000 | 3 | 25.0 | 352.1 | 2.004 | 0.001509 | 0.5313 | 99.5 |
| 0.1000 | 4 | 25.0 | 351.5 | 2.004 | 0.001509 | 0.5304 | 99.3 |
| 0.1000 | 5 | 25.0 | 352.5 | 2.004 | 0.001509 | 0.5319 | 99.6 |
| 0.0100 | 1 | 21.5 | 127.0 | 0.502 | 0.000382 | 0.0485 | 90.8 |
| 0.0100 | 2 | 21.5 | 134.5 | 0.502 | 0.000382 | 0.0514 | 96.3 |
| 0.0100 | 3 | 21.5 | 137.5 | 0.502 | 0.000382 | 0.0526 | 98.5 |
| 0.0100 | 4 | 21.5 | 139.0 | 0.502 | 0.000382 | 0.0531 | 99.5 |
| 0.0100 | 5 | 21.5 | 139.3 | 0.502 | 0.000382 | 0.0532 | 99.6 |

* Since the $a$ volumes of the apparatus were 2.004 and 0.502 instead of exactly 2.000 and 0.500 cc., the factors from Table I are multiplied by $\frac{2.004}{2.000}$ and $\frac{0.502}{0.500}$.

mixture used in the analyses of Table II, the same amounts of acetic acid and $NaNO_2$ are diluted to 8 cc.

*Analysis of Blood Plus Amounts of Amino Acid and Urea.*

Four portions of 10 cc. each of the same blood were placed each in a 100 cc. measuring flask and treated as follows:

*Sample 1.*—The blood was diluted to about 70 cc. 10 cc. each of 10 per cent sodium tungstate and 2/3 N sulfuric acid were added. The volume was made up to 100 cc., and the mixture was filtered after a half-hour.

*Sample 2.*—0.2 cc. of 10 per cent Squibb's urease was added, let stand 1 hour at 22°, then diluted and precipitated with tungstic acid as above.

*Sample 3.*—2 cc. of 1 per cent urea solution (200 mg. of urea = 89.3 mg. of urea N, per 100 cc. of blood) were added; then

TABLE III.

*Analysis of Blood, Blood Plus Urea, and Blood Plus Leucine.*

Urea N = 13 mg. per 100 cc.

| Sample No. | Treatment of blood other than precipitation of proteins. | $P_{N_2}$ at 0.5 cc. volume. | Temperature. | Amino N per 100 cc. blood. |
|---|---|---|---|---|
| | | *mm.* | *°C.* | *mg.* |
| 1 | None. Analyzed by Method A. | 113.7 | 21.5 | 8.67 |
| | | 112.7 | 21.5 | 8.59 |
| | | Average minus 0.07 × urea N . . . . . . . . . . . . . | | 7.72 |
| 2 | Removed urea with urease and magnesia. Analyzed by Method B. | 99.3 | 21.5 | 7.57 |
| | | 97.6 | 21.5 | 7.44 |
| | | Average . . . . . . . . . . . . . . . | | 7.51 |
| 3 | Added 89.3 mg. urea N per 100 cc. Analyzed by Method B. | 100.8 | 21.5 | 7.68 |
| | | 99.8 | 21.5 | 7.51 |
| | | Average . . . . . . . . . . . . . . . | | 7.60 |
| 4 | Added 21.4 mg. amino N per 100 cc. as leucine. Analyzed by Method B. | 380.3 | 21.5 | 28.98 |
| | | 379.5 | 21.5 | 28.92 |
| | | Average . . . . . . . . . . . . . . . | | 28.95 |
| | | Preformed amino N from Sample 2 . . . . . . | | 7.51 |
| | | Added amino N recovered . . . . . . . . . . . . . | | 21.44 |

treated as was Sample 2, except that in precipitating the proteins 11 instead of 10 cc. of 2/3 N sulfuric acid were used to neutralize the ammonia from the added urea.

*Sample 4.*—2 cc. of 1 per cent leucine solution (21.4 mg. of amino N per 100 cc. of blood) were added; then treated as was Sample 2.

Of filtrates of Samples 2, 3, and 4, 20 cc. portions were freed of ammonia by boiling with magnesia, and brought back to 20 cc. volume, as described above, under "Method B" for blood analysis.

The urea content was determined on a separate portion of the blood, and found to be 13.0 mg. of urea N for 100 cc.

In the amino nitrogen determinations of Filtrates 2, 3, and 4, the reaction period was 4 minutes.  In the analysis of Filtrate 1,

TABLE IV.

*Comparison of Methods A and B in Analyses of Normal and Uremic Blood*

| Blood | Method A Correction made for urea | | | | Method B Urea removed | | |
| | $P_{N_2}$ at 0.5 cc volume | Temperature | Amino N per 100 cc blood | | $P_{N_2}$ at 0.5 cc volume | Temperature | Amino N per 100 cc blood |
| | | | Uncorrected | Corrected by subtracting 0.07 urea N | | | |
| | mm | °C | mg | mg | mm | °C | mg |
| 1  Normal   Urea  N  = | 128.8 | 22.0 | 9.80 | 8.93 | 111.3 | 22.0 | 8.47 |
| 12.4 mg  per cent | 127.9 | 22.0 | 9.73 | 8.86 | 113.0 | 22.0 | 8.60 |
| 2  Normal   Urea  N | 124.0 | 20.5 | 9.49 | 9.03 | 115.2 | 23.8 | 8.72 |
| 6.5 mg  per cent | 122.4 | 20.5 | 9.36 | 8.90 | 119.0 | | 9.01 |
| 3  Uremic   Urea  N  − | 205.5 | 24.0 | 15.74 | 7.21 | 93.1 | 21.5 | 7.09 |
| 121.7 mg  per cent | 213.0 | 24.0 | 16.22 | 7.69 | 96.2 | 21.5 | 7.33 |
| | 205.0 | 24.0 | 15.70 | 7.17 | 95.1 | 21.5 | 7.25 |
| | | | | | 94.9 | 21.5 | 7.23 |
| 4  Uremic *  Urea  N  = | 256.0 | 22.0 | 17.96 | 9.42 | 123.2 | 21.2 | 9.39 |
| 122.0 mg  per cent | 256.0 | 22.0 | 17.96 | 9.42 | 122.0 | 21.2 | 9.30 |

* Blood drawn 2 days before death in uremia

where urea was present, the reaction period was made as exactly as possible 3 minutes and 45 seconds, as indicated for 21.5° by the scale of Fig. 4.

It is evident from the results in Table III that the urease treatment of the blood followed by boiling of the filtrate with magnesia satisfactorily removed the urea nitrogen, and that the added amino acid nitrogen was completely recovered.

The margin by which the 7.72 mg. per cent of amino N found

by Method A exceed the 7.5 mg. per cent by Method B (see Sample 2) is about the difference usually noted, as stated above in the description of Method B.

*Comparison of Methods A and B in Analyses of Uremic and Normal Blood.*

The analyses in Table IV illustrate the nature of the results in normal and in uremic blood. Method A is not recommended for uremic blood. However, even in such blood, as shown by Samples 3 and 4, Method A is capable of giving approximate results.

### SUMMARY.

Gasometric determination of primary aliphatic amino nitrogen by the nitrous acid reaction in the manometric apparatus of Van Slyke and Neill is described. The procedure requires the same time, 12 to 15 minutes per analysis in the case of $\alpha$-amino acids, as in the original amino nitrogen apparatus of the writer, and permits measurement of amino nitrogen concentration to 0.0001 mg. per cc., or one-tenth the former limit. In consequence, amino acid nitrogen can be manometrically determined directly in 5 cc. portions of Folin-Wu blood filtrate.

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# THE MANOMETRIC DETERMINATION OF UREA IN BLOOD AND URINE BY THE HYPOBROMITE REACTION.

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The writer has already published (11) gasometric methods for exact estimation of urea in blood and urine by determination of the $CO_2$ formed when urea is converted into ammonium carbonate by the action of urease. This enzyme is probably the most specific reagent we possess for determination of urea, and there is every reason to believe that the results obtained with it are exact within the limits of the methods of measurement used to determine the $NH_3$ or $CO_2$ formed, when the conditions for the action of the urease are properly chosen.[1]

Hypobromite is a much less specific reagent for urea. According to the conditions of the reaction it evolves varying proportions of nitrogen gas from other nitrogenous products, such as uric acid and creatinine. From ammonia it drives off the nitrogen as completely as from urea. With urea itself the reaction does not

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[1] Addis (2) has shown that when extract of jack beans, used as "urease," is mixed with liver tissue, the arginase of the tissue splits urea from some constituent of the extract, and that the yield of ammonia obtained in consequence is manyfold that attributable to the urea content of the liver. He finds that when whole blood is treated for an hour at 38° with a great excess of jack bean extract the same phenomenon occurs to an appreciable extent. Under the conditions of analysis defined by Van Slyke and Cullen (13) and by Van Slyke (11), with action of the urease at room temperature and not unnecessarily prolonged, the reaction discovered by Addis does not enter as a source of error, even when whole blood is treated with the enzyme. As shown recently by the writer (11) the results are the same, under these conditions, as when the enzyme acts on the Folin-Wu filtrate.

yield quite the theoretical amount of nitrogen gas indicated by the equation,

$$CO(NH_2)_2 + 6\,Br + 6\,NaOH = Na_2CO_3 + 6\,H_2O + 6\,NaBr + N_2$$
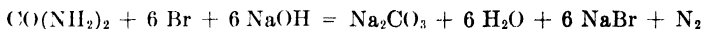
Marie Krogh (5) found that, depending upon the concentrations of NaOH and Br present, the proportion of nitrogen evolved as $N_2$ from urea varied from 86 to 100 per cent, the highest $N_2$ yields being obtained when the least excess of Br was used. The writer has never been able to obtain quite 100 per cent.

Despite the lack of specificity and stoiciometrical precision, the great speed and convenience of the hypobromite reaction and the simplicity of the reagents have caused its survival and have in luced modern investigators (6, 8) to seek conditions under which the drawbacks could be minimized. Thus Marie Krogh (5) precipitated the interfering urinary substances with phosphotungstic acid, and Stehle (8) removed the chief offender, ammonia, with the permutit reagent introduced into analytical chemistry by Folin and Bell (3).

In the procedures for analysis of urine and blood outlined below we have utilized the hypobromite solution previously adapted to gasometric ammonia determination in micro Kjeldahl analyses (9). This reagent with pure urea solutions yields up to 99 per cent of the theoretical amount of nitrogen gas, the yield being higher when the hypobromite is more dilute.

For urine analyses we have followed Stehle (8) in removing ammonia with permutit. When the resultant filtrate is allowed to react with hypobromite for 1 to 2 minutes in the manometric apparatus of Van Slyke and Neill (14) the amount of non-urea nitrogen evolved approximately compensates for the deficit of $N_2$ from urea, as exemplified in Table V. In urines with relatively low percentage of total nitrogen in the form of urea, the $N_2$ from the non-urea substances will somewhat overcompensate for the 5 per cent deficit in the nitrogen gas yield from urea itself, while in urines, such as are obtained with very high protein diets, with relatively high percentages of the total nitrogen in the form of urea, the non-urea substances will fall somewhat short of compensating. A plus or minus error amounting to 4 per cent of the urea present may thus occur. The hypobromite method is not

to be used when such an error would invalidate the interpretation of the results.

In blood filtrates the ammonia is negligible, but the relative proportion of other non-urea nitrogenous substances, not so simply removed, is much greater than in urine. Consequently hypobromite with blood filtrates evolves more nitrogen than is contained in the urea present. Under the conditions outlined below for use of the hypobromite reaction with the Folin-Wu filtrate, the yield of $N_2$ usually indicates in human blood from 1 to 4 mg. more of urea nitrogen per 100 cc. of blood than is present, according to exact analysis with urease. If a correction of 2 mg. is subtracted, the hypobromite nitrogen figure thus corrected falls usually within 1 or 2 mg. of the correct value. Because of the margin of error the hypobromite method is not recommended when the blood urea content is to be compared with the urea excretion rate (1,7), in order to ascertain whether there is a moderate diminution of renal function. The 2 mg. error which may occur may exceed 20 per cent of the blood urea nitrogen, and lead to a corresponding error in the interpretation of the results.

However, the hypobromite blood urea determination is adequate when one wishes only to ascertain whether sufficient urea has been retained to raise the blood level above the maximum normal limit, which MacKay and MacKay (6) put at 23 mg. of urea nitrogen per 100 cc. The hypobromite urea nitrogen is the quickest and simplest of all blood nitrogen determinations, duplicate analyses are easy to repeat with constancy extraordinary for micro analyses, and the determination may well replace that of non-protein nitrogen in laboratories where the latter is a routine procedure for detection of nitrogen retention.

### Reagents.

*Hypobromite Solution.*—To 50 cc. of an alkali solution containing 40 grams of NaOH per 100 cc. add 1 cc. of bromine. The solution is prepared in a 250 cc. Erlenmeyer flask. Before portions are withdrawn for analyses the solution is rotated about the walls of the flask for a half-minute in order to permit the escape into the air of slight amounts of oxygen which form when the reagent

stands. The hypobromite solution should be used the day it is prepared.

*Permutit.*—See Folin and Bell (3).

*Tungstic Acid.*—The sodium tungstate, sulfuric acid, and water, with which Folin and Wu (4) mix blood in order to obtain a protein-free filtrate, can be conveniently combined into a single solution. To 7 volumes of water add 1 volume of 10 per cent sodium tungstate and 1 volume of 2/3 N sulfuric acid. This solution may be used for about 2 weeks. Eventually too much of the tungstic acid settles out as a precipitate, and a fresh solution must be prepared.

### Procedure for Urine Analysis.

1 cc. of concentrated urine (sp. gr. over 1.030), or 2 cc. of more dilute urine, are placed in a 100 cc. Erlenmeyer flask, and either 19 or 18 cc. of water from a burette are added, to make the volume up to 20 cc. 3 grams of permutit are added and the mixture is shaken 4 minutes to remove ammonia. The fluid is then filtered through a dry filter.

1 cc. of water is placed in the cup of the Van Slyke-Neill apparatus. 2 cc. of the urine filtrate are either layered under the water, or are pipetted through it into the chamber of the Van Slyke-Neill apparatus, as shown in Fig. 4 of Van Slyke and Neill's paper (14). After the pipette has been withdrawn, the water is run into the chamber after the urine filtrate and is followed by 1 cc. of the hypobromite solution. The cock is sealed with a drop of mercury. The mercury in the chamber is at once lowered to the 50 cc. mark, and the chamber is shaken, according to the temperature, for 1.5 minutes at 25°, 2 minutes at 20°, or 3 minutes at 15°. The volume of gas is then reduced to 2 cc. and the pressure $p_1$ is read on the manometer.

A blank analysis is run, in which 2 cc. of water, previously shaken with permutit, replace the urine filtrate. The manometer reading is taken as $p_0$.

The chamber of the apparatus need not be washed between the successive analyses of a series. Consequently analyses can be run off at the rate of about one every 4 minutes.

One blank analysis, run at the beginning, serves for an entire series of analyses. If the temperature in the water jacket of the

apparatus rises between the time at which the blank was run and the time of the urine analysis, 1.3 mm. are added to $p_0$ for each degree of temperature increase, to correct for rise in vapor tension; and a similar correction is subtracted from $p_0$ if the temperature falls.

*Calculation of Urine Urea.*—The pressure of $N_2$ is

$$P_{N_2} = p_1 - p_0$$

The urea content of the urine is calculated as:

Per cent of urea or urea nitrogen $= P_{N_2} \times$ factor

The values of the factor are given in Table II.

## Procedure for Blood Urea.

The proteins are precipitated by diluting 1 volume of blood to 10 volumes with the tungstic acid solution. The filtrate is passed through a dry filter.

Of the filtrate 5 cc., equivalent to 0.5 cc. of blood, are pipetted through a mercury seal into the chamber of the Van Slyke-Neill apparatus, in the manner shown in Fig. 3 of a former paper (10). 1 cc. of hypobromite solution is then passed into the chamber in the same manner. The mercury in the cup is then run down into the chamber, only enough remaining above the cock to fill the capillary. The mercury level in the chamber is lowered to the 50 cc. mark, and the chamber is shaken for 1.5 minutes at 25°, 2 at 20°, or 3 at 15°. The reaction must not be allowed to continue longer; slow decomposition of non-urea nitrogenous substances would occasion too high results.

The meniscus of the solution is brought to the 0.5 cc. mark in the chamber in analyses of bloods of urea nitrogen content up to 40 or 50 mg. per 100 cc. For uremic blood the 2.0 cc. mark is used. The reading on the manometer is taken as $p_1$.

A blank analysis is performed with a 5 cc. portion of 0.9 per cent NaCl solution replacing the blood filtrate. The manometer reading is recorded as $p_0$. It is taken with the gas volume at both the 0.5 and 2.0 cc. marks, in order that $p_0$ values shall be available for bloods of either normal or high urea content. The 0.9 per cent NaCl solution has the same solubility for air as the Folin-Wu

filtrate. Hence the blank analysis corrects for dissolved air in the sample of filtrate. In order to make the correction exact, the temperature of the filtrate should differ by not more than 0.2° from that of the 0.9 per cent NaCl. It is advisable to put a 10 or 20 cc. portion of the latter into a 100 or 200 cc. flask, similar to those used for receiving the blood filtrate, before beginning the analyses of a series of filtrates. One ascertains that the temperatures of all are alike, and then rotates them about the walls of their flasks for about a minute, to make certain that they are all saturated with air at the same temperature.

As in the urine analyses, the chamber of the gas apparatus need not be washed out between analyses, so that the latter in series can be run off rapidly.

If the temperature rises during the interval between the blank analysis and the analysis of the blood filtrate, 2.0 mm. are added to $p_0$ for each degree of temperature increase, or subtracted for each degree of temperature fall, when the manometer readings are taken with the gas at 0.5 cc. volume. Of this correction 1.3 mm. are for the change in vapor tension of water in the chamber, and 0.7 mm. for pressure change in the amount of air extracted from the 5 cc. of solution analyzed.

When the manometer readings are taken with the gas at 2.0 cc. volume, the correction to $p_0$ for temperature change after the blank analysis is only 1.5 mm. per degree, the pressure change of the admixed air at the larger volume being only 0.2 mm. per degree of temperature change.

*Calculation of Blood Urea.*—The pressure $P_{N_2}$ of nitrogen gas is

$$P_{N_2} = p_1 - p_0$$
Mg. urea nitrogen per 100 cc. blood $= (P_{N_2} \times \text{factor}) - 2.0$
Mg. urea per 100 cc. blood $= (P_{N_2} \times \text{factor}) - 4.0$

The values of the factors for different room temperatures are given in Table II. The subtraction of 2 mg. of urea N, or 4 mg. of urea, per 100 cc. of blood, from the amount indicated by the nitrogen gas evolved, is to correct for the $N_2$ yielded by non-urea substances of the blood filtrate (see Table VI).

The factors in Tables I and II are calculated as described in the preceding paper on amino nitrogen determination (12), except

that the millimols of $N_2$ gas are multiplied by 28.02 instead of 14.01 to obtain mg. of nitrogen in the sample.  In the factors of Tables I and II no correction is made for the fact that the hypobromite reaction yields only 0.95 mol of $N_2$ per mol of urea under

TABLE I.

*Factors by Which Millimeters $P_{N_2}$ Are Multiplied to Give Urea Nitrogen and Urea Contents of Sample Analyzed.*

| Temperature. | Factors giving mg. urea N. | | Factors giving mg. urea. | |
|---|---|---|---|---|
| | $a = 0.5$ cc.* | $a = 2.0$ cc. | $a = 0.5$ cc. | $a = 2.0$ cc. |
| °C. | | | | |
| 15 | 0.000780 | 0.003121 | 0.001671 | 0.00669 |
| 16 | 77 | 10 | 66 | 67 |
| 17 | 75 | 0.003099 | 60 | 64 |
| 18 | 72 | 87 | 54 | 62 |
| 19 | 69 | 76 | 48 | 60 |
| 20 | 67 | 65 | 42 | 57 |
| 21 | 64 | 54 | 36 | 55 |
| 22 | 61 | 43 | 30 | 53 |
| 23 | 59 | 33 | 24 | 50 |
| 24 | 56 | 22 | 19 | 48 |
| 25 | 53 | 11 | 13 | 46 |
| 26 | 50 | 01 | 07 | 44 |
| 27 | 48 | 0.002990 | 01 | 41 |
| 28 | 45 | 79 | 0.001596 | 39 |
| 29 | 43 | 69 | 90 | 37 |
| 30 | 40 | 59 | 85 | 35 |
| 31 | 37 | 49 | 80 | 32 |
| 32 | 35 | 39 | 74 | 30 |
| 33 | 32 | 29 | 69 | 28 |
| 34 | 30 | 18 | 63 | 26 |

* $a$ indicates the volume at which gas pressure in the Van Slyke-Neill apparatus was read.

the conditions of urine analysis, and 0.98 mol under the conditions of blood analysis.  The deficit of $N_2$ yield from the urea is compensated by $N_2$ from other substances in the urine, and more than compensated in the blood.

TABLE II.

*Blood and Urine Analyses.*

*Factors by Which Millimeters $P_{N_2}$ Are Multiplied to Give Urea Nitrogen and Urea Content.*

| Temperature. | Factors for urine analyses. | | | | Factors for blood analyses.* | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Giving gm urea N per 100 cc. | | Giving gm. urea per 100 cc. | | Giving mg. urea N per 100 cc. Sample = 0.5 cc. blood. | | Giving mg. urea per 100 cc. Sample = 0.5 cc. blood. | |
| | Sample = 0.1 cc. urine. (a=2.0 cc ) | Sample = 0.2 cc. urine. (a=2.0 cc.) | Sample = 0.1 cc. urine. (a=2.0 cc ) | Sample = 0.2 cc. urine (a=2.0 cc.) | a = 0.5 cc. | a = 2.0 cc. | a = 0.5 cc. | a = 2.0 cc. |
| °C. | | | | | | | | |
| 15 | 0.00312 | 0.001561 | 0.00669 | 0.00335 | 0.1561 | 0.624 | 0.335 | 1.336 |
| 16 | 11 | 55 | 67 | 34 | 55 | 22 | 34 | 31 |
| 17 | 10 | 49 | 64 | 32 | 49 | 20 | 32 | 26 |
| 18 | 09 | 44 | 62 | 31 | 44 | 18 | 31 | 22 |
| 19 | 08 | 38 | 60 | 30 | 38 | 15 | 30 | 17 |
| 20 | 07 | 33 | 57 | 29 | 33 | 13 | 29 | 13 |
| 21 | 05 | 27 | 55 | 28 | 27 | 11 | 28 | 08 |
| 22 | 04 | 22 | 53 | 27 | 22 | 09 | 27 | 03 |
| 23 | 03 | 16 | 50 | 25 | 16 | 06 | 25 | 1.298 |
| 24 | 02 | 11 | 48 | 24 | 11 | 04 | 24 | 94 |
| 25 | 01 | 06 | 46 | 23 | 06 | 02 | 23 | 90 |
| 26 | 00 | 00 | 44 | 22 | 00 | 00 | 22 | 85 |
| 27 | 0.00299 | 0.001495 | 41 | 21 | 0.1495 | 0.598 | 21 | 80 |
| 28 | 98 | 90 | 39 | 20 | 90 | 96 | 20 | 76 |
| 29 | 97 | 85 | 37 | 19 | 85 | 94 | 19 | 72 |
| 30 | 96 | 80 | 35 | 18 | 80 | 92 | 18 | 67 |
| 31 | 95 | 74 | 32 | 16 | 74 | 90 | 16 | 62 |
| 32 | 94 | 69 | 30 | 15 | 69 | 88 | 15 | 58 |
| 33 | 93 | 64 | 28 | 14 | 64 | 86 | 14 | 54 |
| 34 | 92 | 59 | 26 | 13 | 59 | 84 | 13 | · 50 |

* To approximate the true urea content of blood, subtract from the blood urea values calculated by the above factors, 2 mg. of urea N or 4 mg. of urea per 100 cc. of blood, as correction for the $N_2$ yielded by non-urea substances in blood filtrate. No correction is required for urine urea values calculated by the above factors.

## EXPERIMENTAL.

### Reaction of Urea with Hypobromite under Conditions of Urine Analysis.

Urea solutions of 1 and 3 per cent concentration were analyzed as above outlined for urines, with the exception that in some of the determinations the treatment with permutit was omitted, in order to ascertain whether the reagent has any effect on urea. The results in Table III show that the $N_2$ yield approximated 95 per cent of theoretical, and that permutit has no effect whatever.

TABLE III.

*Hypobromite Reaction with Urea under Conditions of Urine Analysis.*

1 cc. hypobromite + 2 cc. 20-fold diluted solution + 1 cc. water.

| Concentration of urea solutions before dilution. | Permutit used or not. | Time of reaction with hypobromite. | $P_{N_2}$ $a = 2$ cc. | Temperature. | Urea found. | Per cent of urea present found by analysis. |
|---|---|---|---|---|---|---|
| *gm. per 100 cc.* | | *min.* | | *°C.* | *gm. per 100 cc.* | |
| 2.97 | 0 | 1 | 433.2 | 23.5 | 2.818 | 94.8 |
| | 0 | 2 | 436.0 | 23.5 | 2.830 | 95.3 |
| 2.97 | + | 1 | 434.5 | 23.0 | 2.824 | 95.1 |
| | + | 2 | 435.0 | 23.0 | 2.828 | 95.2 |
| 0.99 | 0 | 1 | 144.4 | 23.5 | 0.937 | 94.6 |
| | 0 | 2 | 145.2 | 23.5 | 0.942 | 95.2 |
| 0.99 | + | 1 | 143.5 | 23.0 | 0.933 | 94.2 |
| | + | 2 | 144.5 | 23.0 | 0.939 | 94.8 |

### Reaction of Urea with Hypobromite under Conditions of Blood Analysis.

Solutions of urea were prepared simulating in concentration those observed in normal and nephritic human blood. These solutions were diluted 10-fold, and were analyzed as described above for Folin-Wu blood filtrate, in all details except one; the blank analysis for determination of the $p_0$ value was performed with water instead of 0.9 per cent NaCl solution, since in these analyses the urea solutions were free from salts. The results in

Table IV indicate that approximately 98 per cent of the theoretical amount of $N_2$ is evolved.   The 1 cc. of hypobromite solution in this case is diluted with 5 cc. of urea solution, instead of the 3 cc. added in the urine analyses.   The greater dilution of the alkaline hypobromite apparently increases the yield of $N_2$ from the urea.

TABLE IV.

*Hypobromite Reaction with Urea under Conditions of Blood Analysis.*

5 cc of 10-fold diluted urea solution + 1 cc. hypobromite.   Reaction for 2 minutes

| Urea added to solution | Concentration of urea N in solution,* before dilution. | $P_N$. | Volume of gas when $P_{N_2}$ was measured. | Temperature | Urea N found. | Proportion of urea N present found by the analysis. |
|---|---|---|---|---|---|---|
| *mg  per 100 cc* | *mg. per 100 cc.* | *mm.* | *cc.* | *°C.* | *mg. per 100 cc.* | *per cent* |
| 20 | 9.24 | 60.0 | 0.5 | 22.2 | 9.13 | 98.9 |
|  |  | 59.6 | 0.5 | 22.2 | 9.07 | 98.2 |
| 50 | 2.31 | 149.9 | 0.5 | 22.2 | 22.8 | 98.7 |
|  |  | 147.6 | 0.5 | 22.2 | 22.5 | 97.4 |
|  |  | 148.2 | 0.5 | 22.2 | 22.5 | 97.4 |
| 100 | 4.62 | 297.3 | 0.5 | 22.2 | 45.2 | 97.8 |
|  |  | 295.0 | 0.5 | 22.2 | 44.9 | 97.2 |
|  |  | 299.0 | 0.5 | 22.2 | 45.5 | 98.5 |
| 200 | 9.24 | 147.5 | 2.0 | 22.2 | 89.7 | 97.1 |
|  |  | 148.2 | 2.0 | 22.2 | 90.1 | 97.6 |
|  |  | 148.5 | 2.0 | 22.2 | 90.3 | 97.8 |

* The N content of the urea used was found by Kjeldahl analyses to be 46.20 instead of the theoretical 46.62 per cent.

*Urine Analyses.*—The results obtained in urine analyses are exemplified in Table V.   It is evident that the non-urea substances continue reacting after the 1 minute which suffices for the maximum yield of $N_2$ from urea.   The rate, however, at which $N_2$ is evolved from these substances after the first minute is relatively so slow that the increase per minute is less than 1 per cent of the $N_2$ evolved in the first minute.   In the time that we have used for the reaction the amount of nitrogen from the non-urea substances approximately compensates for the deficit of 5 per cent in the nitrogen evolved from the urea itself.

*Blood Analyses.*—The results recorded in Table VI are from human blood, partly normal, partly from nephritic subjects with nitrogen retention. The hypobromite analyses were performed

TABLE V.

*Urine Analyses.*

*Urea N by Hypobromite Method.*

| Urine No. | Urine sample represented in portion of filtrate analyzed. | Time of reaction with hypobromite. | Temperature. | $P_{N_2}$ (a = 2 cc.) | Urea N content of urine found by hypobromite. | Urea N content by urease method. |
|---|---|---|---|---|---|---|
| | *cc.* | *min.* | *°C.* | *mm.* | *per cent* | *per cent* |
| 1 | 0.1 | 1 | 24.0 | 179.4 | 0.542 | |
| | 0.1 | 2 | 24.0 | 180.9 | 0.546 | 0.524 |
| | 0.1 | 3 | 24.0 | 182.5 | 0.551 | |
| 2 | 0.1 | 1 | 24.5 | 160.2 | 0.483 | 0.492 |
| | 0.1 | 2 | 24.5 | 162.0 | 0.489 | |
| | 0.1 | 3 | 24.5 | 163.8 | 0.494 | |
| 3 | 0.1 | 1 | 25.0 | 397.5 | 1.197 | 1.208 |
| | 0.1 | 2 | 25.0 | 400.5 | 1.206 | |
| | 0.1 | 3 | 25.0 | 403.5 | 1.215 | |
| 4 | 0.1 | 1 | 25.0 | 285.0 | 0.858 | 0.869 |
| | 0.1 | 2 | 25.0 | 286.0 | 0.861 | |
| | 0.1 | 3 | 25.0 | 287.0 | 0.864 | |
| 5 | 1.0 | 1 | 25.0 | 314.0 | 0.095 | 0.098 |
| | 1.0 | 2 | 25.0 | 321.0 | 0.097 | |
| 6 | 0.2 | 1 | 25.0 | 234.0 | 0.352 | 0.366 |
| | 0.2 | 2 | 25.0 | 235.0 | 0.354 | |
| 7 | 0.2 | 1 | 25.0 | 559.0 | 0.842⎫ | 0.860 |
| | 0.1 | 1 | 25.0 | 279.8 | 0.842⎭ | |
| | 0.2 | 2 | 25.0 | 561.0 | 0.845⎫ | |
| | 0.1 | 2 | 25.0 | 281.8 | 0.848⎭ | |

as outlined above, the urease determinations by the gasometric determination of the $CO_2$ of the ammonium carbonate formed, as described in a previous paper (11).

TABLE VI.

*Blood Analyses.*

| Blood No. | Urea N by urease method.<br>(a) | Hypobromite N, total uncorrected.<br>(b) | Excess hypobromite N over urea N.<br>(b) − (a) | Difference between corrected hypobromite N* and urea N.<br>((b) − (a) − 2) |
|---|---|---|---|---|
| | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* |
| 1 | 6.8 | 9.0 | 2.2 | +0.2 |
| 2 | 8.5 | 11.0 | 2.5 | +0.5 |
| 3 | 8.7 | 11.0 | 2.3 | +0.3 |
| 4 | 8.8 | 9.0 | 0.2 | −1.8 |
| 5 | 9.3 | 9.7 | 0.4 | −1.6 |
| 6 | 9.6 | 12.1 | 2.5 | +0.5 |
| 7 | 11.1 | 13.1 | 2.0 | 0.0 |
| 8 | 11.3 | 13.7 | 2.4 | +0.4 |
| 9 | 11.4 | 13.9 | 2.5 | +0.5 |
| 10 | 12.6 | 16.0 | 3.4 | +1.4 |
| 11 | 12.7 | 13.9 | 1.2 | −0.8 |
| 12 | 15.0 | 14.6 | 0.4 | −2.4 |
| 13 | 15.3 | 15.8 | 0.5 | −1.5 |
| 14 | 15.6 | 19.5 | 3.9 | +1.9 |
| 15 | 17.0 | 20.2 | 3.2 | +1.2 |
| 16 | 18.4 | 20.7 | 2.3 | +0.3 |
| 17 | 18.9 | 20.0 | 1.1 | −0.9 |
| 18 | 19.0 | 20.9 | 1.9 | −0.1 |
| 19 | 19.3 | 20.9 | 1.6 | −0.4 |
| 20 | 19.5 | 23.3 | 3.8 | +1.8 |
| 21 | 22.5 | 26.1 | 3.6 | +1.6 |
| 22 | 26.1 | 26.6 | 0.5 | −1.5 |
| 23 | 27.2 | 29.5 | 2.3 | +0.3 |
| 24 | 31.4 | 35.1 | 3.7 | +1.7 |
| 25 | 38.7 | 42.3 | 3.6 | +1.6 |
| 26 | 57.3 | 58.0 | 0.7 | −1.3 |
| 27 | 57.7 | 61.6 | 3.9 | +1.9 |
| 28 | 58.7 | 57.9 | 0.8 | −2.8 |
| 29 | 71.1 | 69.5 | 1.6 | −3.6 |

* Corrected hypobromite N = (total observed) − (2 mg. per 100 cc.).

### SUMMARY.

Procedures are described for the approximate determination of urea in urine and in the Folin-Wu blood filtrate by the hypobromite reaction with the Van Slyke-Neill manometric gas apparatus. 3 minutes suffice for the gasometric determination. The results can be utilized when a maximum error is permissible of 1 part in 25 of urine urea, or of 2 mg. of blood urea nitrogen per 100 cc. In blood analyses the method is adequate to ascertain whether the urea concentration is within the limits of normal variation, and affords a rapid and simple substitute for non-protein nitrogen estimation as an indicator of nitrogen retention.

The analyses presented above were in part performed by Mr. John Plazin.

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# THE STORAGE OF MANGANESE AND COPPER IN THE ANIMAL BODY AND ITS INFLUENCE ON HEMOGLOBIN BUILDING.*

By R. W. TITUS

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The controversy relative to the amount of time necessary to produce a secondary type of anemia in young animals suggested to us the idea of not only a prenatal supply of iron in the young animal but also a supply of certain other accessory elements. The fact that young rats from mothers on a whole wheat-milk powder diet became anemic much more rapidly than young rats from mothers on a more complex ration, indicated to us that the question might be very largely the prenatal supply or storage of the hemoglobin-building elements.

In a previous paper (1) Titus, Cave, and Hughes presented data to show that not only copper but also manganese is effective in the utilization of iron in hemoglobin building. In this paper we are presenting data to substantiate our claim that manganese, as well as copper, may be stored in the animal body in such a way as to be effective in the utilization of iron in hemoglobin building.

### EXPERIMENTAL.

Young rats, about 4 weeks of age, were taken from their mothers and placed in individual galvanized iron wire cages. These ani-

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* The analytical work for this paper was done at the Kansas State Agricultural College.

The analytical data included in this paper were presented before the Biological section of the American Chemical Society at the Columbus meeting in May, 1929.

mals were fed whole cow's milk *ad libitum* plus a supplement. Lot I (Fig. 1) received for 5 weeks, in addition to the milk, a supple-
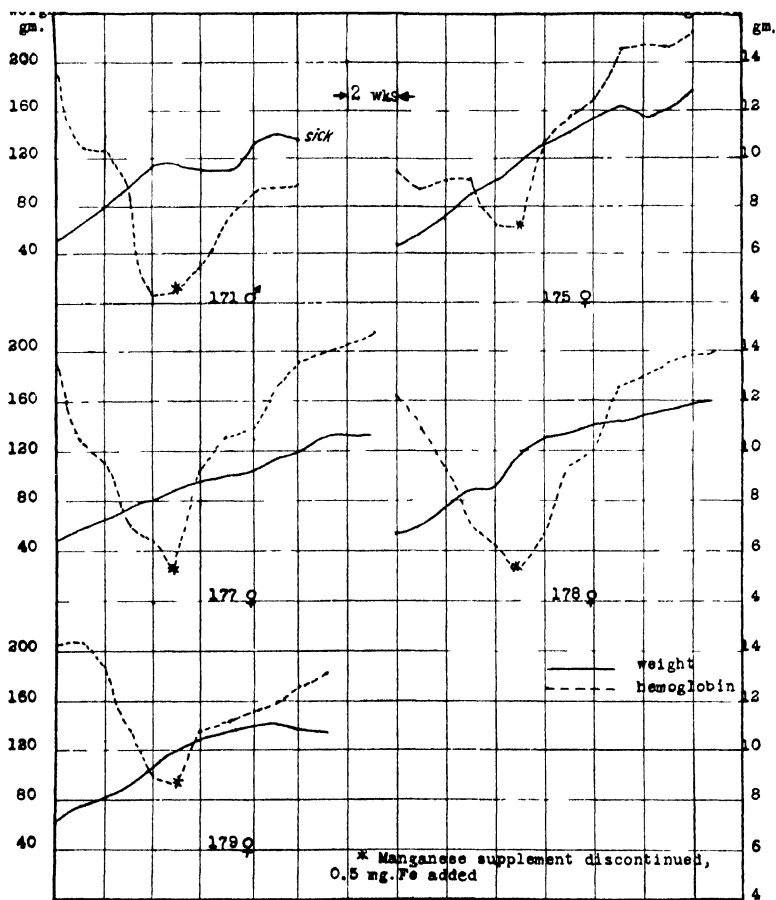


FIG. 1. Lot I. Growth and hemoglobin curves for rats on an iron supplement following manganese supplement.

ment of 0.1 mg. of manganese in the form of the chloride. At the end of this period the manganese supplement was discontinued and a supplement of 0.5 mg. of pure iron, in the form of the chloride, was added.

Lot II (Fig. 2) received for 5 weeks, in addition to the milk, a supplement of 0.05 mg. of copper in the form of the sulfate. At
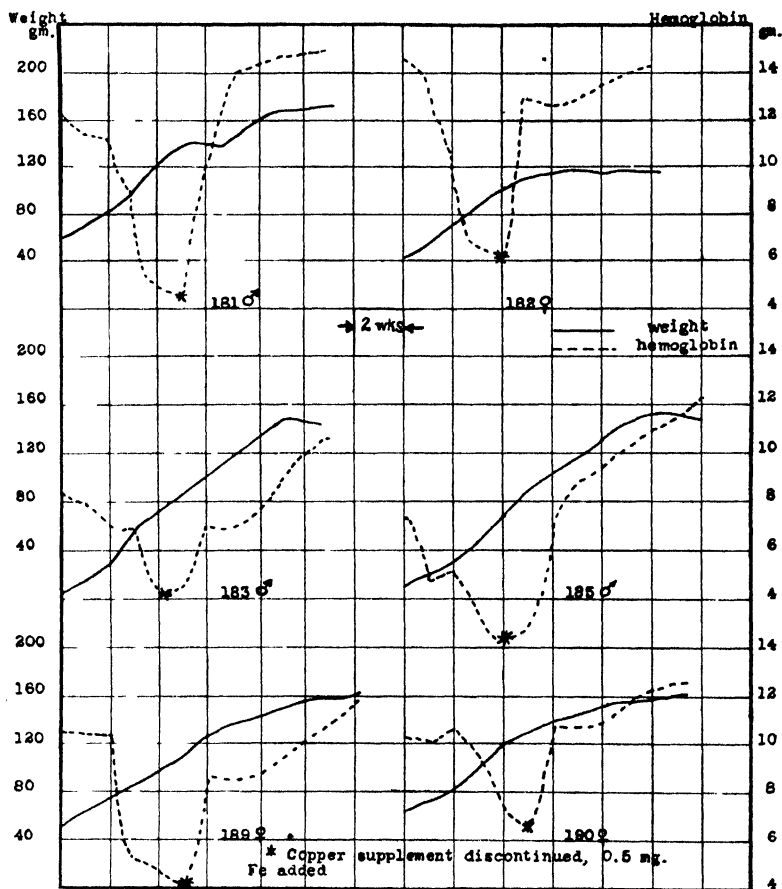


FIG 2  Lot II  Growth and hemoglobin curves for rats on an iron supplement following copper supplement.

the end of this period the copper supplement was discontinued and a supplement of 0.5 mg. of pure iron was added.

Lot III (Fig. 3) received for the entire experimental period, in

addition to the whole milk diet, 0.5 mg. of pure iron, in the form of
the chloride.
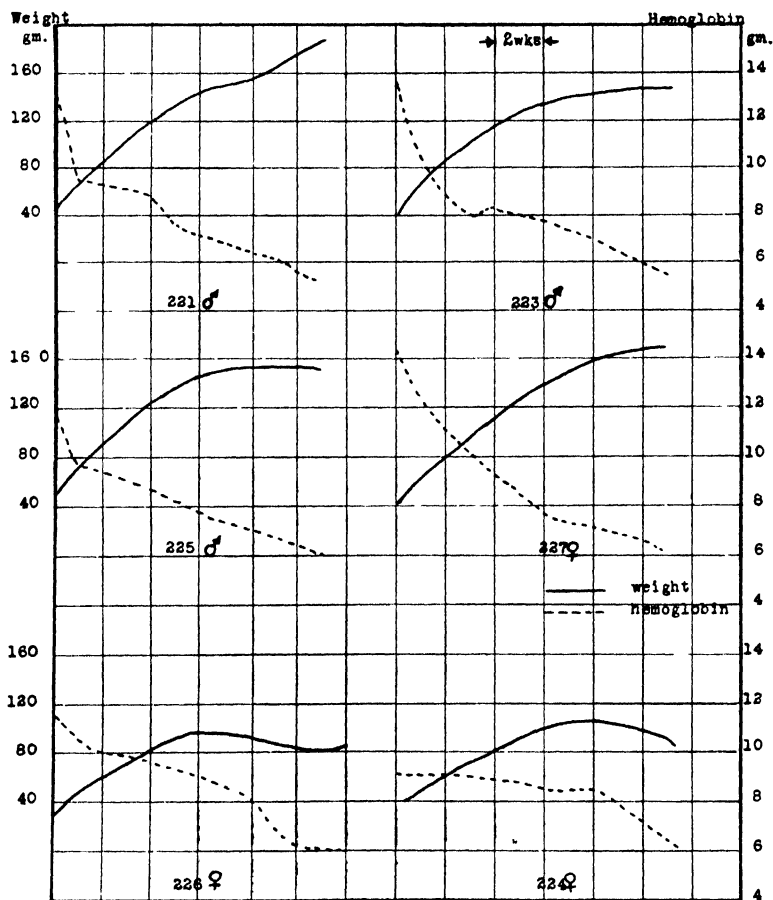


Fig. 3. Lot III.   Growth and hemoglobin curves for rats on a milk diet
+ pure iron supplement.

The animals in Lots I and II showed a gradual decrease in the
hemoglobin content of their blood until the time the manganese or
the copper supplement was discontinued.   At this point the iron
supplement was added and the hemoglobin content of the blood

immediately began to increase until in about 5 weeks it was practically normal. The animals in Lot III gradually became anemic, although it required a longer time to produce the same degree of anemia than it did when no iron was added in a supplementary way. It is possible that the animal is able to utilize a small amount of iron, due, not to copper or manganese as an impurity in the food, but rather to a prenatal storage of these elements in the body.

The mineral supplement was so made up that the daily supplementary dose was dissolved in 1 cc. of doubly distilled water and added to the liquid whole milk in clean porcelain mortars. The data for the experimental work are shown in graphic form in Figs. 1 to 3.

### CONCLUSIONS.

1. Both manganese and copper are apparently stored in the animal body when these mineral supplements are added to the ration.

2. Manganese, as well as copper, when fed or stored in the animal body, is effective in the utilization of iron in hemoglobin building.

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# COMPARISON OF BIOLOGICAL AND COLORIMETRIC ASSAYS FOR VITAMIN A AS APPLIED TO FISH OILS.

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Various color tests have been proposed during the past few years for vitamin A; a list of many of the reagents tried may be found in an article by Cocking and Price (1) and elsewhere in the literature. While many of the color reactions have been shown not to be proportional to the vitamin content of an oil, all evidence so far published seems to indicate that the color produced with arsenic trichloride, proposed by Rosenheim and Drummond (2), and with the chloroform solution of antimony trichloride, proposed by Carr and Price (3), is associated with the vitamin A content of the oil tested. However no exact data are available to show a quantitative relationship between the color produced by either of the above reagents and feeding experiments on the oils.

The present work was undertaken to study the color reaction of a series of six fish body and fish liver oils with antimony trichloride and attempt to correlate the color with careful feeding experiments which had been made on each oil.

Wokes and Willimott (4) have pointed out that certain plant pigments also produce a blue color when treated with antimony trichloride or arsenic trichloride. Therefore in the presence of plant pigments these reactions may not be used.

In order to correlate the colorimetric with the biological test, Rosenheim (5) suggests the use of 20 mg. of cod liver oil as the standard amount for analysis, which he calls the animal unit, for he found that this amount of average cod liver oil will induce a weekly increase of 8 to 10 gm. in rats kept on a diet otherwise free from vitamin A. The color produced when treated with 1 cc. of the arsenic or antimony trichloride reagent is taken as the color unit

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equivalent to 1 animal unit.    This amount of oil they state, gives
a blue color which, when measured in a 10 mm. cell, matches 10
blue units of Lovibond standards.

Wokes and Willimott (6) in order to show the effect of varying
amounts of oils with a given amount of reagent chose three potent
cod liver oils.    Their curves show that in higher concentrations
the color produced is not proportional to the amount of oil used
in the test and the rate of flattening of the curve is not the same
for the different oils.    However they state that in lower concen-
trations the curves are linear and suggest that in running a test
such amount of oil be taken as to give a reading   below 18 units of
blue when measured in a half inch cell.    In another paper (4)
they state that the results cease to be linear functions above a
concentration of 2 to 3 per cent and therefore the amount of oil
taken for a test should be such that the color produced when read
in Lovibond units will not be higher than 15 to 20 units.    From
our results it will be shown that such concentrations cannot be
used as a basis for quantitative comparison of the factor which
causes the production of color when treated with antimony tri-
chloride reagent.

### Experimental Method.

The method used is a modification of that used by other work-
ers (6-8).    A solution of antimony trichloride in chloroform was
prepared by dissolving 30 gm. of fresh Baker's analyzed antimony
trichloride in 100 cc. of chloroform at room temperature.    Be-
fore being used, the reagent was cooled to the temperature of ice
water, about 2-4°; for, as pointed out by Wokes and Willimott (4),
at ordinary room temperature, i.e. 22-25°, the color changes so
rapidly it is difficult to get an accurate reading, and below 15°
the reagent is clouded if saturated at room temperature.    How-
ever if the reagent is cooled to the temperature of ice water and
allowed to remain until equilibrium is reached, a clear solution is
obtained containing about 18 per cent (weight volume per cent)
of antimony trichloride; thus permitting the use of lower tem-
peratures than room temperature under ordinary laboratory con-
ditions, for a long enough period to obtain an accurate reading.
As shown by Wokes and Willimott (6) the intensity of the color
produced varied with different strengths of reagents.    But the

TABLE I.

*Color Produced by Varying Concentrations of Various Oils when Treated with Antimony Trichloride Reagent.*

| Mg. oil added in 0.3 cc. solution to 3.0 cc. reagent. | Color in Lovibond units. | | |
|---|---|---|---|
| | Blue. | Yellow. | Red. |
| Cod Liver Oil A. | | | |
| 0.69 | 1.7 | 0.4 | |
| 1.38 | 2.8 | 0.6 | |
| 2.76 | 5.0 | 1.0 | |
| 5.4 | 7.5 | 1.5 | |
| 13.8 | 14.0 | 2.4 | |
| 27.6 | 18.5 | 2.0 | |
| 55.5 | 27.0 | 5.0 | |
| Ratfish liver oil. | | | |
| 5.4 | 0.7 | 0.3 | 0.2 |
| 13.5 | 1.3 | 0.6 | 0.4 |
| 27.0 | 2.0 | 0.9 | 0.6 |
| 67.5 | 3.2 | 1.4 | 0.9 |
| 135.0 | 5.2 | 2.3 | 1.1 |
| 270.0 | 7.0 | 3.0 | 1.2 |
| Chinook salmon body oil. | | | |
| 67.4 | 1.7 | 2.2 | 1.3 |
| 137.1 | 3.0 | 5.6 | 3.5 |
| 274.2 | 4.0 | | 6.0 |
| Sockeye salmon body oil. | | | |
| 138 | 1.5 | 1.7 | 0.8 |
| 273 | 2.7 | 2.8 | 1.3 |
| Silver salmon body oil. | | | |
| 219 | 1.4 | 2.1 | 0.9 |
| 276 | 2.0 | 2.5 | 0.9 |
| Humpback salmon body oil. | | | |
| 207 | 0.9 | 2.5 | 1.4 |
| 276 | 1.8 | 4.0 | 3.0 |

conditions as here described are easily reproducible and therefore give results which are comparable.

By means of a 1 cc. serological pipette 0.3 cc. of the oil or solution of oil in chloroform to be tested was introduced into a half inch cell and the cell placed in position in the Lovibond tintometer. 3 cc. of the cooled antimony trichloride reagent were then added
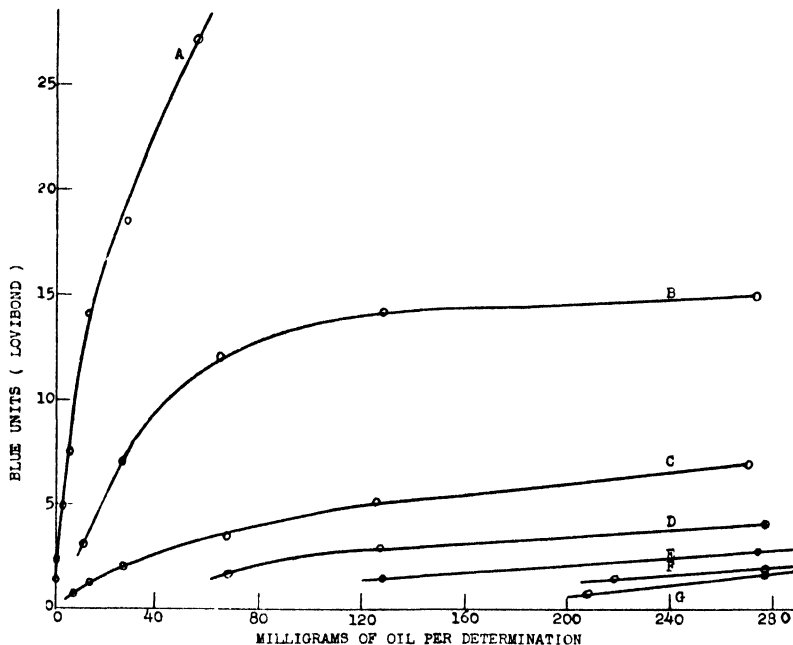


FIG. 1. Intensity of color plotted against mg. of oil used. A indicates Cod Liver Oil A; B, Cod Liver Oil B; C, ratfish liver oil; D, Chinook salmon body oil; E, sockeye salmon body oil; F, silver salmon body oil; G, humpback salmon body oil.

from a 3 cc. pipette, mixing being accomplished by delivering the reagent in a strong stream into the cell.    After allowing 10 to 15 seconds to pass in order to have the maximum color develop, the color of the solution was matched with standard Lovibond units of blue, Series 1180, yellow, Series 510, and red, Series 200, and a reading taken at the end of 30 seconds.

A series of dilutions of cod liver oils, ratfish liver oil, Chinook,

sockeye, silver, and humpback salmon body oils was prepared
in chloroform.   The color produced when 0.3 cc. of each dilution
was mixed with 3 cc. of the reagent was measured.   Each of the
color values recorded in Table I is the average of four to six
separate tests.
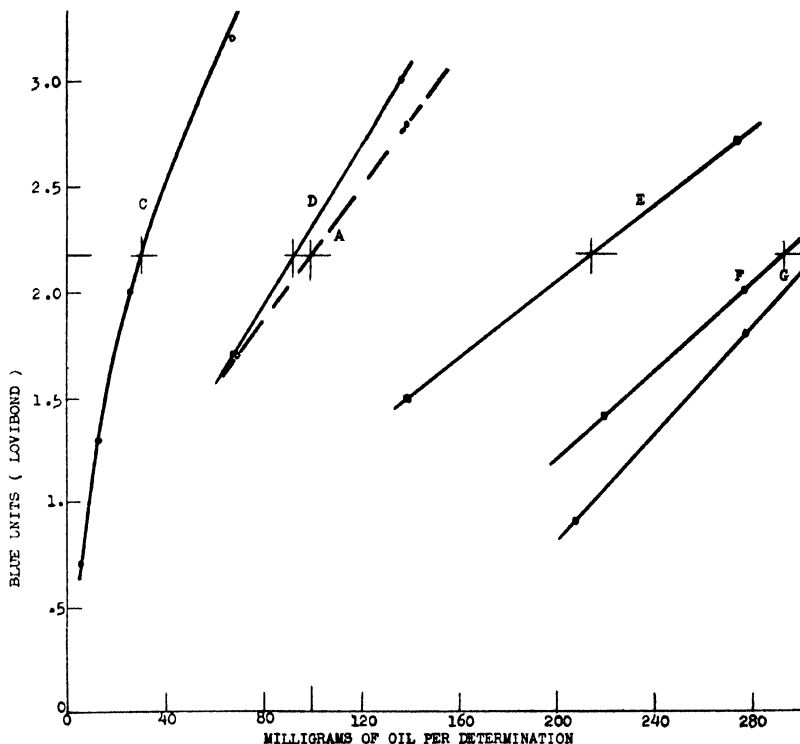


FIG. 2. Comparison of color values made by plotting lower values on a
larger scale against the mg. of oil used.   Letters have the same significance
as in Fig 1

When the intensity of the color produced is plotted against
the mg. of oil used in the reaction, a smooth curve is obtained as
shown in Fig. 1.   In no case is it found that the color produced
is directly proportional to the amount of oil used and therefore
is not directly proportional to the vitamin A content, if the color
reaction is produced by this factor.   At no concentration of cod

liver oil is the curve linear.   Consequently a direct comparison cannot be made between two cod liver oils at color values as high as 10 blue units as suggested by Rosenheim or 15 to 20 units as suggested by Wokes and Willimott, as will be evident from Fig. 1.

However the curves approach a straight line at very low color values.   By interpolation the color value equivalent to 1 animal unit of Cod Liver Oil A (0.00099 gm.) when used in a color determination as described above is found to be 2.18 Lovibond blue units.   The animal unit is taken as that amount required daily to cause a growth of 25 gm. in 8 weeks, following the method of Sherman and Munsell (9).   The amount of each oil necessary to give the same color, when the same technique is used, may be inter-

TABLE II.

*Comparison of Amounts of Oil Required to Give 1 Animal Unit As Determined by Biological and Colorimetric Assay.*

| Name of oil. | Biological assay. | Colorimetric assay. |
|---|---|---|
| | *gm. per day* | *gm. per day* |
| Cod Liver Oil A*. ........  ................ | 0.00099 | Standard |
| Ratfish liver oil. ........................... | 0.029 | 0.03 |
| Chinook salmon body oil*................ . .... | 0.086 | 0.09 |
| Sockeye salmon body oil* ................... | 0.160 | 0.21 |
| Silver salmon body oil*.   .................... | 0.247 | 0.29 |
| Humpback salmon body oil*. .  .............. | 0.320 | 0.31 |

* Biological assay determined by Boynton and Truesdail.

polated.   Or by plotting the lower values on a larger scale against the mg. of oil used per determination as in Fig. 2 a comparison of the color values may be made.   Cod Liver Oil A is plotted on a scale of the abscissa 100 times greater than the other oils and is used as a basis for comparison.   The amount of each oil required to give the same color as that obtained with 0.00099 gm. of cod liver oil may be read on the abscissa.   These values, given to the nearest 10 mg., agree fairly well with those obtained by biological assay as shown in Table II.   The biological assay of the ratfish liver oil was made by Norris and Danielson (10).   The biological assay of the oils marked with an asterisk in Table II were determined by Boynton and Truesdail (11).

## SUMMARY.

1. The blue color produced by a fish oil and antimony trichloride reagent is not proportional to the amount of oil used.

2. At no concentration is the curve obtained with varying amounts of cod liver oil in antimony trichloride reagent linear.

3. The colorimetric assay upon the fish oils tested checks within reasonable limits with the biological assay when the technique described in this paper is used.

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# AN ELECTRON TUBE POTENTIOMETER FOR THE DE-
# TERMINATION OF pH WITH THE GLASS
# ELECTRODE.

By WILLIAM C. STADIE.

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Recent developments (Clark, 1928) of the glass electrode indi-
cate that it might well become the method of choice for the deter-
mination of pH in serum, blood, etc. Its usefulness is seriously
restricted, however, by the fact that the internal resistance of the
glass cell is high (20 to 500 megohms) necessitating the use of a
troublesome quadrant electrometer in place of the customary
galvanometer. The elimination of the electrometer would ap-
preciably enhance the value of the method. This paper de-
scribes an easily controlled, sensitive, and stable electron tube
potentiometer which will measure an E.M.F. to 0.001 volt or less
through resistances up to 600 megohms. No electrostatic effects
are present, body and hand capacity effects are negligible, the
indicating galvanometer image is extremely steady, and the sen-
sitivity with the Leeds and Northrup galvanometer No. 2420C is
1 to 4 mm. per 0.001 volt. With glass electrodes of 30 to 50
megohms resistance filled with buffers, equilibrium is quickly
obtained and maintained constant ($\pm$0.0005 volt) for 2 to 8 hours.

Several types of electron tube potentiometers have been de-
scribed (Clark, 1928), but these have been used exclusively with
cells of low internal resistance. Recently, however, two papers
have appeared (Elder and Wright, 1928; Partridge, 1929) which
describe potentiometers for use with cells of high internal resistance.
The method described here is different in principle from those re-
ported and possesses certain advantages which will be discussed
below.

*Determination of Plate Current-Grid Potential Characteristics of*
*Electron Tubes.*

Fig. 1 shows the customary circuit by which the properties of the
tube are obtained. The electron tube has three electrodes:
filament, grid, and plate. When the filament is heated (by the A
battery) current flows in three circuits; *viz.*, filament circuit and
(by virtue of the electron emission from the hot filament) the grid
and plate circuits. The negative filament terminal $(A-)$ is the
point common to the three circuits and all potential differences
are referred to it as zero.
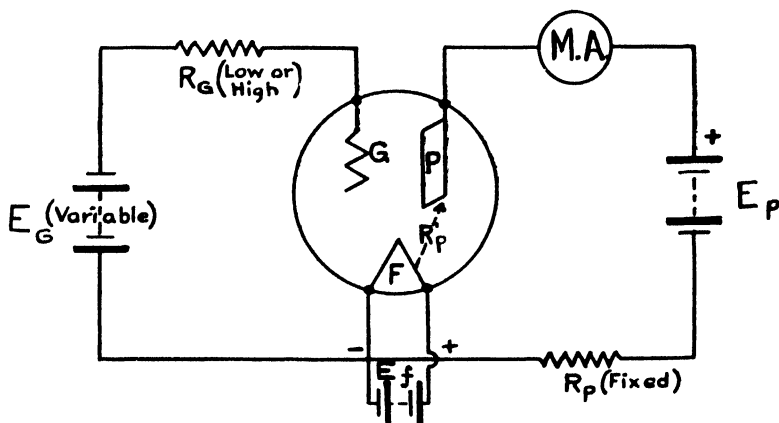


FIG 1. Circuit to determine grid potential-plate current characteristics
of electron tubes.

Let　　　　　　$E_p$ = plate potential (high and positive)
　　　　　　　$I_p$ = the plate current
　　　　　　　$R_p$ = the external plate resistance
　　　　　　　$R'_p$ = the internal plate resistance

The plate current is given by the equation

$$I_p = \frac{E_p}{R_p + R'_p} \tag{1}$$

For any given tube the plate current depends on the plate poten-
tial, the filament temperature, the external and internal plate resist-
ance, the grid potential, and the external grid resistance. We

discuss only the effects of variations of the grid potential ($E_g$) and the external grid resistance ($R_g$) on the plate current, all other factors affecting $I_p$ remaining constant. Changing $E_g$ from a negative to a positive value decreases the internal plate resistance ($R'_p$) and hence increases $I_p$ (Equation 1). The curve of $I_p$ plotted against $E_g$ is the *plate current-grid potential characteristic* or briefly the *characteristic*. Two cases arise: (1) The resistance in the grid circuit ($R_g$) is low. This is the familiar case and is illustrated by the solid line in Fig. 2. Only the straight portion of the curve
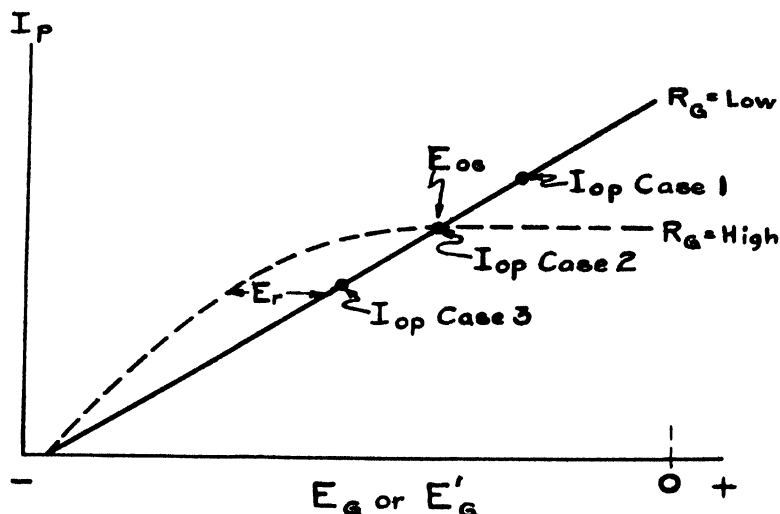


Fig. 2. Characteristics of electron tube when external grid resistance is low or high. The characteristic with high external resistance is distorted due to residual gas in the tube.

concerns us. Its slope $\dfrac{\Delta I_p}{\Delta E_g}$ or the *amplification* depends on the tube, plate voltage, etc. We may take $0.3 \times 10^{-6}$ ampere per millivolt as an average value for the electron tubes suitable for use as laboratory potentiometers. $\dfrac{\Delta I_p}{\Delta E_g}$ is the change of plate current which must be measured to determine an E.M.F. to 0.001 volt. (2) The resistance in the grid circuit is high ($R_g$) > 2 megohms. The presence of small irremovable traces of gas

within the tubes produces a distortion of the characteristic when a high resistance is placed in the grid circuit. All tubes tested (Nos. 199, 171, 201A, 222, 300A) gave altered characteristics of the same general nature. A knowledge of the nature of the curves permits the selection of suitable conditions for operating the tube as a voltmeter in measuring the E.M.F. of glass cells. Denote grid potentials on the high resistance curves by $E'_g$. The curve (Fig. 2) is convex to the straight line characteristic between $E'_g = -8.0$ to $-1.0$ volts but is almost parallel to it between $E'_g = -3.0$ to $-1.0$ volts. Above $-1.0$ volt the curve passes through the low resistance characteristic at a point whose abscissa is always negative. At this point the plate current is the same as that when the grid circuit is open, which is equivalent to making $R_g = \infty$. The curve then becomes asymptotic to a slightly higher value of $I_p$. On higher plate voltages (67.5 to 180 volts) the convexity of the high resistance characteristic is more marked. Moreover the position of the characteristic varies with the resistance. We further consider the plate current $(I_{og})$ when the grid circuit is opened, $i.e.$ when $(R_g = \infty)$. Its value is not that at 0 grid potential (grid to $A-$ when $R_g = 0$) but less. In other words the open grid acts as if it had a potential negative to $A-$. Call this apparent potential the $open$ $circuit$ $grid$ $potential$ $(E_{og})$. It varies from $-0.2$ to $-0.8$ volts in the tubes tested ($-4$ volts in tube No. 202A).

The two curves (high and low grid resistances) intersect at a point whose coordinates are $I_{og}$ and $E_{og}$. $E_{og}$ is constant for any set of conditions but varies with filament temperature, plate potentials, etc. Note that if $E_g = E'_g = E_{og}$ the grid may be placed on low, high, or infinite grid resistance without change in plate current. Denote the horizontal distance between the curves by $E_r$. To the left of $E_{og}$, $E_r$ is negative and small; to the right $E_r$ is positive and large. Any selected and constant value of $I_p$ intersects the curves at points called the operating points $(I_{op})$ and the grid potentials $E_g$ and $E'_g$ for low and high grid resistances respectively at the same $I_{op}$ are obviously related by

$$E'_g = E_g + E_r$$

The terms defined are collected in tabular form for reference.

*Symbols.*

$I_p$ = plate current in general

$I_{op}$ = plate current at any selected operating point

$I_{og}$ = plate current when grid is on open circuit

$E_g$ = grid potential when external grid resistance is low

$E_g'$ = grid potential when external grid resistance is high

$E_{og}$ = abscissa of point of intersection of characteristics with high and low external grid resistance

$E_r$ = horizontal distance between two characteristics

$E_x$ = E.M.F. of unknown in grid circuit

$E'$ = E.M.F. of compensating potentiometer in grid circuit

$\dfrac{\Delta I_p}{\Delta E_g}$ = increment of plate current to increment of grid potential or amplification

$R_g, R_p$ = external resistance in grid and plate circuits respectively

$R_p'$ = internal resistance of filament to plate

### E.M.F. *Measurement by Electron Tubes.*

The electron tube may be used in two ways to measure E.M.F. The first is the *deflection method.* The plate current-grid potential characteristic is established by calibration with known E.M.F. in the grid circuit. An unknown E.M.F. in the grid circuit may be then read directly from the resulting $I_p$ and this curve. The tube characteristic, however, is subject to frequent changes and the variation of $I_p$ over a considerable range introduces serious variations in the tube action. Since the characteristic varies with the resistance this method is not suitable for use with glass cells.

The second or *null method* possesses the advantage that the tube action is maintained constant by keeping $I_p$ at some selected operating point. Call this constant plate current $I_{op}$. An unknown E.M.F. $= E_x$ and the E.M.F. $= E'$ of a compensating potentiometer in series are placed in the grid circuit. The plate current may be restored to the operating point by adjustment of the potentiometer. When the grid resistance under both conditions is low it is clear from Fig. 2 that no extra grid potential is needed so that $I_{op}$ is constant when $E_x = E'$.

When, however, the unknown has a high resistance as in the case of a glass cell an additional negative E.M.F. $= E_r$ must be put on the grid to maintain $I_{op}$ constant. Obviously $E_r = E_x + E'$ from which

$$E_x = E_r - E' \tag{2}$$

$E_r$ depends on resistance of the glass cell and the operating conditions (Fig. 2) of the tube, but its exact value 'need not be known since it is constant for any given cell if the operating conditions are constant.    Since all pH measurements with the glass electrode are relative to some known pH, Equation 2 and the familiar Nernst equation give for any two solutions the equation

$$E_{x_1} - E_{x_2} = E_2' - E_1' = k\frac{RT}{NF}\,(\mathrm{pH_1} - \mathrm{pH_2})$$

from which

$$\mathrm{pH_1} = \mathrm{pH_2} + \frac{NF}{kRT}\,(E_2' - E_1')$$

$\mathrm{pH_2}$ being known, $\mathrm{pH_1}$ may be calculated from the difference of the potentiometer readings.   The absolute value of $E_x$ can be calculated only if the cell resistance and value of $E_r$ at this resistance is known. The need for this in most pH work is rare.

The significance of the characteristics with low and high grid resistances in the measurement of the E.M.F. of glass cells by the null method is brought out by the following remarks.   Select some fixed grid potential ($E_g$) by introducing a dry cell into the grid circuit and maintain the filament temperature constant.   $I_{op}$ and $E_r$ are thus fixed.   Connect the grid (always through the source of grid potential $E_g$) by a switch (1) directly to $A$ — through low resistance or (2) indirectly through the E.M.F. $= E_x$ of the glass cell (which includes a high resistance) and the E.M.F. $= E'$ of a compensating potentiometer.   In passing from low to high resistance there will be no change of plate current when $E_r = E_x + E'$. Fig. 2 makes it clear that by variation of the fixed grid potential ($E_g$) relative to the open grid potential ($E_{og}$) three cases arise.

|  | Case 1. $E_g > E_{og}$ | Case 2. $E_g = E_{og}$ | Case 3. $E_g < E_{og}$ |
|---|---|---|---|
| $E_r$........................ . | Positive and infinite. | Zero. | Negative and small. |
| Amplification $\left(\frac{\Delta I_p}{\Delta E_g}\right)$..... | Zero. | Small. | Large. |
| Grid current............ ...... | Large. | Zero. | Zero. |

The schema shows whether and how the current can be restored to the operating point in the three cases.   Case 1 is completely

excluded since it would be impossible to restore the plate current to the operating point. The second use has the advantage that at balance the grid can be connected to $A-$ directly or through the glass cell or be on open circuit without change in plate current. However the difficulty of exactly locating the $E_{og}$, the diminished amplification, and the possibility of grid current flowing through the cell if $E_{og}$ is not exactly located nullify this advantage. Case 3 $(E_g < E_{og})$ by a simple arrangement yields maximum sensitivity and retains all the stability of Case 2. $E_{og}$ for tube UX 222 varies from $-0.2$ to $-0.5$ volt depending on operating conditions. A fixed bias $(E_g)$ of $-1.5$ volts gives proper working conditions for this tube.

## Grid Current.

In the measurement of any reversible E.M.F. it is of course essential that no current be drawn from the cell. In galvanometer circuits of high sensitivity this current may be reduced to very low values usually of the order of $10^{-10}$ to $10^{-8}$ amperes. A perfect electron tube should have no grid current at a bias of 0 volt (grid to $A-$) but again the presence of gas alters its action so that, in general, tubes uniformly show zero grid circuit at $E_g = E_{og}$ rather than $E_g = A-$. Grid current increases rapidly as $E_g$ becomes less negative so that at $E_g = A-$ there is appreciable current. At $E_g$ more negative than $E_{og}$ ($-0.5$ to $-3$ volts) no grid current can be demonstrated by a galvanometer whose sensitivity is $2 \times 10^{-8}$ amperes per mm. If the grid bias is made highly negative ($-3$ to $-6$ volts) there is a small (about $10^{-9}$ amperes) grid current in the *opposite* direction. This is a third gas effect. The working condition of $-1.5$ volt for fixed grid bias assures zero current taken from the cell.

## Practical Electron Tube Potentiometer Circuits.

Both deflection and null methods require current indicators in the plate circuit with high sensitivity ($0.3 \times 10^{-6}$ amperes to measure 0.001 volt) and considerable range (since $I_p$ may be 0.5 to 1.5 milliamperes). These requirements cannot be contained in one instrument. The methods commonly used to overcome this difficulty are of five types:

### 1. Use of High Sensitivity Galvanometer with Shunt (*Elder and Wright, 1928*).

A resistance is shunted across the galvanometer and is adjusted to retain the galvanometer image on the scale. Since only a portion of the current passes through the galvanometer, part of the amplification factor of the tube is sacrificed.

### 2. Diminution of Plate Voltage.

This reduces the plate current and lessens the galvanometer deflection at the operating point (Pope and Gowlett, 1927). Again, however, the marked reduction of plate voltage sacrifices amplification.

### 3. Increase of Negative Grid Potential (*Williams and Whitenack, 1927*).

The plate current may be reduced to zero at sufficiently negative grid potential (Fig. 2) at the cost, however, of considerable amplification.

### 4. Multiple Stage Amplification.

The above methods are difficult in practice and the use of the galvanometer directly in the plate circuit has generally been abandoned. To employ a milliammeter one or two further stages of amplification may be added to the first tube and the amplification factor $\frac{\Delta I_p}{\Delta E_g}$ increased say 20-fold. An instrument sensitivity of about $6 \times 10^{-6}$ then suffices to measure a change of E.M.F. in the grid of 0.001 volt so that a milliammeter with range of 1.50 milliamperes for 150 scale divisions may be used (Partridge, 1929; Goode, 1928).

### 5. Balanced Wheatstone Bridge Circuit.

Multistage circuits designed to increase amplification require large plate voltages with heavy drain on B batteries. The author has found that under these circumstances there is unsteadiness of plate current requiring frequent adjustments of the operating point. These difficulties have been fully discussed (Goode, 1928; Bienfait, 1926) and have led the author to abandon the method for a simpler one which completely overcomes all the

difficulties discussed and permits the use of a·high sensitivity galvanometer indirectly in the plate circuit.

The principle of the circuit is that of a Wheatstone bridge with arms containing four resistances, two of which are the internal (filament to plate) resistances of the tubes; and two are the external (variable) resistances of the plate circuit. These latter when properly selected balance the bridge. A high sensivitity galvanometer across the bridge measures zero current at balance and if the electron tubes possess approximately the same electrical properties, changes of filament and plate batteries affect the internal resistances of both tubes alike so that balance is not dis-
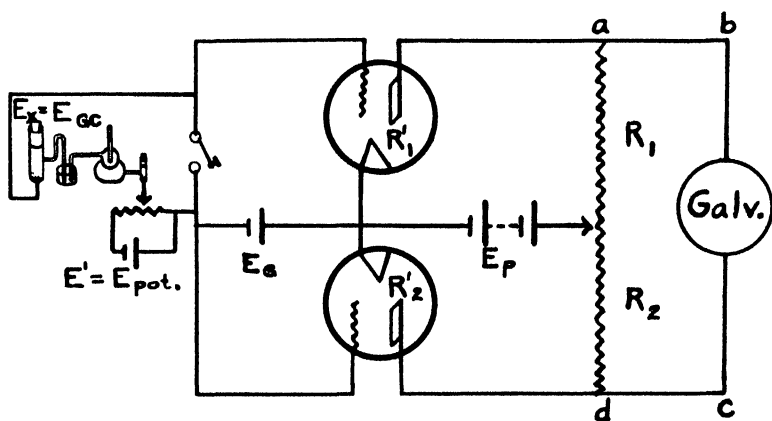


Fig. 3. Schematic balanced Wheatstone bridge circuit adapted to measure E.M.F. of the glass electrode in one grid circuit.

turbed. By this arrangement great steadiness of tube action is obtained independent of minor fluctuations of the batteries and the advantage of using a sensitive indicating galvanometer working over a narrow range is secured. The Wheatstone bridge principle in electron tube circuits has been employed in a variety of ways notably in the strobodyne method of stabilizing radio frequency amplification and in push-pull audio-frequency amplification. For laboratory purposes Fitch (1927) has successfully used it in measuring impedance at high frequencies and Wynn Williams (1928) has recently described such a circuit suitable for the measurement of small ionization currents.

The schematic circuit is shown in Fig. 3. (The filament battery is omitted.) The three circuits (grid, plate, and filament) of two electron tubes are connected in parallel and have the usual common C (grid), B (plate), and A (filament) batteries. The grid circuit of one tube (upper) is fitted with a switch allowing a glass electrode and compensating potentiometer to be inserted in series in the circuit at will. If $R_1$ and $R_2$ are the external resistances of the plate circuit (which may be varied) and $R'_1$ and $R'_2$ the internal resistances (filament to plate) of the electron tubes since the circuit is obviously a Wheatstone bridge there will be no current in the circuit abcd when

$$\frac{R_1}{R_2} = \frac{R'_1}{R'_2}$$

If the grid potential on the upper tube is changed by inserting the glass cell and potentiometer, E.M.F., $E$, and $E'$, and $R'_1$ changes, the bridge is unbalanced and a deflecting current will pass through the circuit abcd. This may be reduced to zero by adding $E_r$ volts to the grid potential of the upper tube and we may calculate pH as before. This circuit permits a high sensitivity galvanometer to be inserted in abcd since at balance only small currents are measured and the circuit used as a null method to measure E.M.F. in the grid circuit. With a Leeds and Northrup galvanometer No. 2420C of $0.025 \times 10^{-6}$ ampere per mm. sensitivity a change of $E_g = 0.001$ volt may be made to give a deflection of 4 mm. when the grid resistance is 30 to 600 megohms.

The circuit gives a convenient null method adaptable to the glass electrode. The construction is simple and except for the potentiometer and galvanometer, which are as a rule standard laboratory equipment, is inexpensive. It obviates the disadvantage of multistage amplification and the need for relatively inaccurate and expensive milli or micro ammeters.

Fig. 4 shows in detail the complete circuit used. A standard Weston cell is included so that the potentiometer may be adjusted from time to time. Fig. 5 shows the assembled apparatus. A list of component parts is given.
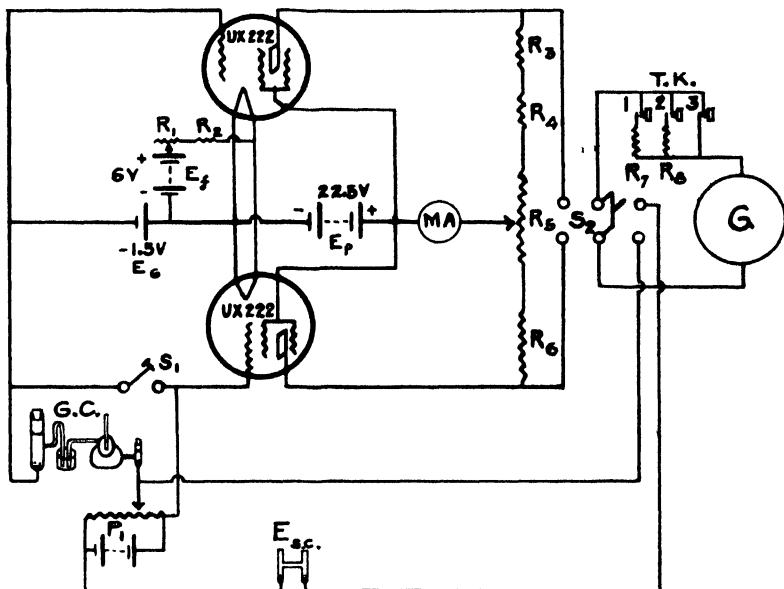
Fɪɢ 4 Working balanced Wheatstone bridge circuit arranged as a potentiometer for use with the glass electrode
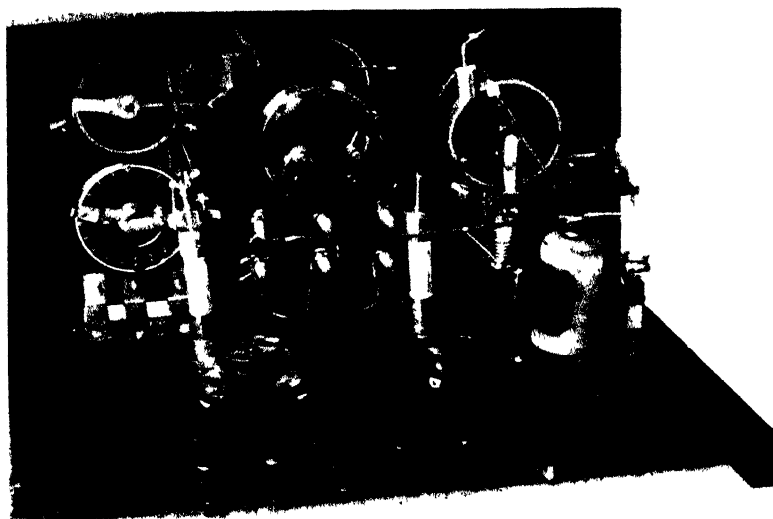


Fɪɢ 5. Assembled electron tube potentiometer for the measurement of ᴇ ᴍ ꜰ. through high resistance, particularly the glass electrode. The electron tubes are omitted.

*Components*

   2    U X 222
   $E_f$    4 to 6 volt storage battery
   $E_g$    1 5 volt flash light cell
   $E_p$    22 5 volt radio battery (heavy duty)
  $E_c$    Weston standard cell
   $R_1$    5 ohm rheostat (Yaxley)
   $R_2$    15 ohm fixed resistance (Yaxley)
$R_3, R_5$    10,000 ohm fixed resistance (Durham 2 5 watt Powerohm)
   $R_4$    400 ohm vernier variable resistance (Yaxley potentiometer)
$R_7, R_8$    fixed resistance 0 5, 0 1 megohms respectively (Durham, Powerohm)
   $P_1$    Leeds and Northrup student potentiometer
   $R_9$    6000 ohm potentiometer (Centralab No P F )
   $S_1$    mercury cup "telegraph key" switch, silica insulated
   $S_2$    double pole-double throw switch (Leeds and Northrup type K)
 $T K$    tapping keys
 $G C$    glass cell and connecting calomel cells
   $G$    Leeds and Northrup galvanometer No 2420C
 $M A$    Weston panel milliammeter (1 5 m Amp No 506)

## *Selection and Matching of Tubes.*

The screen grid D.C. electron tube, UX 222, is superior to any tube tried. The control grid terminal is at the top of the tube and is hence effectively insulated.

A further advantage of the UX 222 tube is the space charge grid which is operated at $+22.5$ volts. With $+22.5$ volts on the plate and filament at 1.5 volts (0.20 ampere for 2 tubes) a plate current of 0.45 millampere and a galvanometer deflection of 3 to 4 mm. per 0.001 volt change of $E_g$ is obtained. The low plate voltage and filament temperature give great steadiness of tube action.

The chief object of the bridge circuit is to reduce fluctuations of galvanometer current due to changes in the tubes or batteries. Theoretically, if both tubes have the same electrical properties, changes in either A or B batteries would affect both alike so that no change in galvanometer current would occur. Practically this is almost accomplished. To insure a stabilized circuit the tubes selected must be matched. The plate current-grid volt characteristics of a batch of tubes are determined and the most closely matching pair selected.

### Insulation.

The internal resistance of the glass electrode varies from 20 to 500 megohms. Obviously the insulation of the grid from A or B battery terminals must be of the highest order. To obtain this the following procedures are necessary.

The control grid is insulated by coating the tube with paraffin. The tube sockets are painted with paraffin. The mounting panel and base of the apparatus are of Bakelite. All wire is of covered bus wire and arranged so that the wires of different circuits do not touch. The A and B batteries are placed on paraffin blocks. The C battery (1.5 volt pocket flash light cell) is paraffined.

The make and break switch is a platinum wire dipping into a small cup of glass containing mercury. The cup is supported by a silica rod. The platinum wire is insulated by a silica rod and is raised and lowered by an arrangement similar to a telegraph key. The leads from the key to the electrode and potentiometer carried through the case are supported in silica tubes. The glass electrode and connecting calomel cells are insulated by silica plates or rods. The potentiometer is placed on insulating blocks.

### Body Capacity Effects and Shielding.

Without adequate grounding violent body and hand capacity effects may be evident even if insulation is most thorough. They can be almost completely eliminated by placing the entire apparatus on a sheet of galvanized iron which is grounded to a water pipe. In addition a sheet of grounded galvanized iron may be placed under the chair of the operator.

No shielding is necessary as the apparatus is practically free of electrostatic effects

### Switching in the Glass Electrode.

Since Case 3 is selected as the working condition the disadvantage of a change of $I_p$ on open grid circuit must be overcome. The importance of this lies in the following: If constant current at the operating point be momentarily altered (as by opening the grid circuit) it will not return instantly to its former value but will fluctuate unsteadily for 2 to 3 minutes and may change so that many adjustments may be necessary before a satisfactory read- ·

ing can be obtained. To eliminate this difficulty no change should
be allowed to take place in the $I_{op}$ and this is accomplished by
never allowing the grid to be on open circuit. To do this the un-
known is shunted across the switch. The grid circuit is short-
circuited on the make and is not influenced by the cell and poten-
tiometer but on the break the grid is connected through the
unknown and potentiometer with zero time of open circuit. It is
possible then to pass freely back and forth from low to balanced
high resistance with negligible variations of $I$.

The disadvantage of short-circuiting the glass cell is slight.
Our experience shows that with selected glass cells polarization
even with a large E.M.F. difference is quickly recovered from.
Polarization is minimized by reducing the time of short-circuit-
ing the cell.

### *Necessity for Constant Plate Current.*

Equation 2 requires $E_r$ to be constant between successive deter-
minations of pH. Now $E_r$ increases as $I_p$ is increased (*e.g.* by
change of filament temperature or plate voltage). In general
the variation of $E_r$ with changes of plate current is greatest when
filament temperature and plate voltage are high. For example,
filament voltage $= 4.0$ and filament current $= 0.3$ ampere, screen
grid and plate voltage $= +45$; the change of $E_r$ is 0.1 volt when $I_p$
changes from 0.90 to 1.10 milliampere or 0.005 volt per 0.01 milli-
ampere. With the conditions selected, however, namely 22.5
volts on plate and screen grid, 1.5 volts on filaments and
plate current $= 0.30$ milliampere, the change of $E_r$ for change
of $I_p$ of 0.01 milliampere is $< 0.001$ volt. Changes in $E_r$ are thus
easily avoided by keeping the current constant by means of the
filament rheostat and the panel milliammeter which reads to 0.01
milliampere.

### *Technique.*

Adjust the filament till $I_p = 0.30$ milliampere and allow 20 min-
utes for the tubes to reach a steady state. Adjust the plate resist-
ances until the galvanometer registers no deflection. Break the
direct grid connection by means of the switch thus putting the
glass electrode and potentiometer in series with the C battery
and grid of the control tube. Rapidly adjust the potentiometer

until the galvanometer is again balanced. Leave the key *open* for 1 to 2 minutes, close the key, and adjust to galvanometer zero a second time. Again insert the unknown and adjust the potentiometer to zero deflection. Repeat at intervals of a minute until the system attains equilibrium. Always maintain plate current constant when the key is closed by filament adjustment. Close the switch when changing the cells for if the grid is left on open circuit for a few minutes the galvanometer zero may shift and may require 3 to 5 minutes to become constant.

## Calculation.

The glass cell constant $e_o$ is first established. Determine the E.M.F. ($E$) of five or six buffers of known pH over the desired range. Plot the value of pH against $E$, draw the best straight line, and obtain the constants of the equation

$$\text{pH} = \frac{NF}{kRT} (E - e_0)$$

The constants $e_o$ and $\frac{NF}{kRT}$ allow the calculation of an unknown pH from its $E_x$ and this equation. In general $\frac{NF}{kRT}$ is close to 0.058 the theoretical value at room temperature and is very constant ($\pm 0.0001$) for any given cell. $e_o$ is not quite so constant so it is best to verify its value daily by determination on one or two known solutions.

### SUMMARY.

A null method for the measurement of E.M.F. of glass electrodes with an electron tube potentiometer is described. The grid, filament, and plate circuits of two electron tubes (No. UX 222) are connected in parallel. The plate circuits are arranged as a Wheatstone bridge which may be balanced by variable resistances allowing a high sensitivity galvanometer to be inserted into the plate circuit. The E.M.F. of a glass cell in the grid circuit of one tube may then be measured to 0.001 volt or less. The apparatus is free from electrostatic disturbances and is very steady in its action. In measuring E.M.F. through resistances of 20 to 600 megohms its sensitivity is 1 to 4 mm. of deflection per 0.001 volt.

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# HEAT AND ULTRA-VIOLET IRRADIATION AS MEANS OF DIFFERENTIATING VITAMINS B AND G IN YEAST.*

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Since it is now a generally accepted fact that vitamin B, the antineuritic factor, and vitamin G, the antipellagric factor are both necessary for growth, it has become important to devise an accurate and at the same time a practical method for isolating each factor. It is also highly desirable to be able to estimate the amount of both factors in the same food material. The destruction of vitamin B by heat is believed to leave vitamin G unimpaired, although conclusive evidence of its complete stability is lacking. It is clearly desirable to have at least as satisfactory a method for destroying vitamin G and retaining the antineuritic vitamin.

Recently Hogan and Hunter (1) have proposed what seemed to be a promising method for destroying the antipellagric vitamin and leaving intact the antineuritic vitamin in yeast. They found that yeast which had been irradiated under controlled conditions would relieve young pigeons from an acute attack of polyneuritis but would not prevent final collapse. This yeast did not promote growth in young rats. From these results they concluded that they had destroyed some factor for growth. As autoclaved yeast which possesses no antineuritic potency corrected the deficiency of the irradiated yeast, Hogan and Hunter concluded that their results gave a means of preparing the antineuritic factor free from other growth-promoting factors.

In continuing our investigations on yeast (2) as a source of the growth-promoting factors, we have not been able to substantiate

these results of Hogan and Hunter. Using the laboratory technique and methods for the preparation of food materials described in a former paper (3), we have tested irradiated yeast as a source of the growth-promoting factors, vitamins B and G, both alone and in conjunction with autoclaved yeast.

A dry starch-free yeast[1] was exposed for $10\frac{1}{2}$ hours, at a distance of 7 inches and in a thin (1 to 2 mm.) layer, to a mercury vapor quartz lamp[2] operating at 110 volts and 5 amperes. The yeast was remixed and respread at frequent intervals. This yeast was made into tablets weighing 0.5 gm. and fed separately from the ration. It was also fed in 0.5 gm. tablets mixed with an equal weight of yeast which had been autoclaved[3] to destroy the antineuritic factor. A ration consisting of purified casein 18 parts, salt mixture (4) 3.7 parts, agar-agar 2 parts, butter fat 9 parts, and tapioca dextrin to make 100 parts was fed to the rats in Lots 1 to 4, and the same ration, except that the dextrin carried the alcoholic extract of ether-extracted wheat embryo equivalent to 15 parts of embryo, was fed to the rats in Lots 5 and 6.

Fig. 1 gives the composite growth curves of the feeding trials which were undertaken to determine whether irradiation was a reliable means of destroying the growth-promoting factors in yeast other than the antineuritic factor. The control group of rats, Lot 1, received daily 0.5 gm. of untreated dry starch-free yeast to supply both factors, vitamins B and G, those of Lot 2 autoclaved yeast, those of Lot 3 irradiated yeast, and those of Lot 4 0.5 gm. of a mixture of equal parts by weight of autoclaved and irradiated yeast. Of these four groups, Lot 3 was the only one which did not respond to its yeast supplement as expected. If irradiation had completely destroyed the antipellagric factor, the rate of growth of these rats would not be as good as represented by these graphs. It is very probable that irradiation and autoclaving impair in varying degrees both vitamin B and vitamin G. Otherwise the rate of growth of the rats in Lot 4 should be as good as that of the rats in Lot 1.

[1] This yeast was purchased from the Northwestern Yeast Company, Chicago.

[2] Hanovia Alpine sun lamp.

[3] From 250 to 300 gm. of dry yeast were autoclaved in a Mason jar for $2\frac{1}{2}$ hours under 15 pounds pressure.

We also fed the irradiated yeast as a supplement to one of our highly purified rations (Lot 5). Although this ration contained
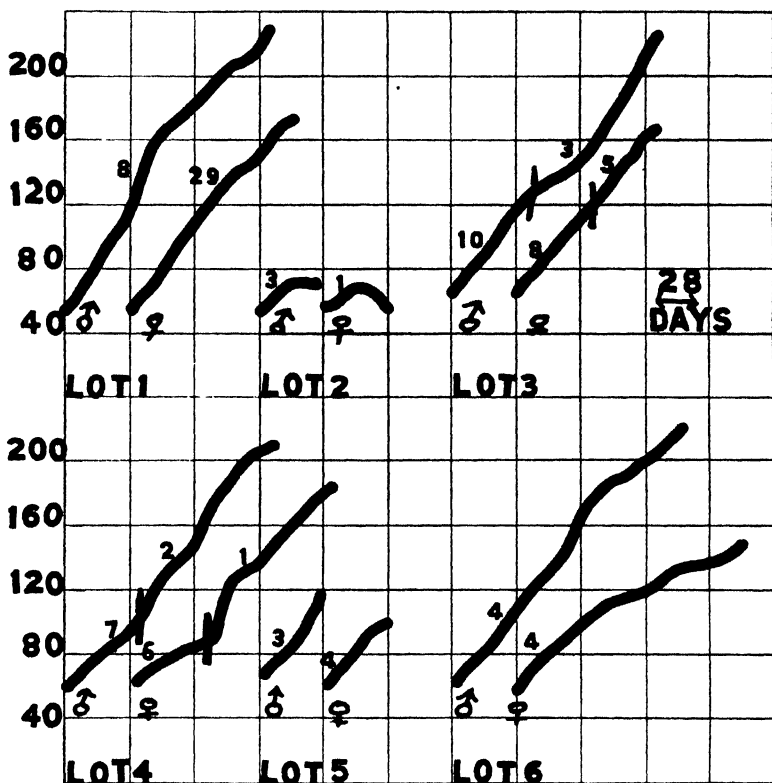


FIG. 1. Composite growth curves of male and female rats, showing the effect of irradiation on the growth-promoting property of yeast. The numbers to the left of the curves designate the number of animals used. Each of the rats received 0.5 gm. of the following yeast preparations as a source of vitamin B or G: Lot 1 starch-free, air-dried yeast, Lot 2 autoclaved yeast, Lots 3 and 5 irradiated yeast, Lots 4 and 6 equal parts of irradiated and autoclaved yeast. The ration of Lots 5 and 6 differed from that of the other lots in that it contained the alcoholic extract of 15 gm of ether-extracted wheat embryo.

what we supposed to be an ample amount of vitamins B and G, it had repeatedly failed to promote growth but had become growth-

promoting when supplemented with small quantities of autoclaved yeast. Since Lot 5 shows that this ration also became growth-promoting when supplemented with the irradiated yeast and as an additional supplement of autoclaved yeast (Lot 6) did not enhance the rate of growth over that of the irradiated yeast alone, we have further evidence that irradiation cannot be relied upon completely to destroy the growth-promoting factors of yeast other than the antineuritic factor.

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# QUANTITATIVE STUDIES OF RESPONSES TO DIFFERENT INTAKES OF VITAMIN D.*

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Present methods for determining vitamins quantitatively depend upon the reproducible and measureable effects occurring in standardized animals, as a result of controlling their intake of the vitamin in question within definite limits. For the study of vitamin D, two types of basal diets are in common use: those which are drastically rickets-producing, owing to decidedly unsatisfactory relations of calcium and phosphorus, as well as to absence or shortage of the vitamin; and those which are deficient in vitamin D but are otherwise adequate. The effects of these diets are reported to include retardation of growth, an interference with ossification, a lowering of the concentrations in the blood serum of calcium or the phosphate ion, or both, and an increase in the alkalinity of the intestinal contents. Different investigators attach varying degrees of significance to each of these phenomena.

From a study of rats on a rickets-producing basal diet, with or without supplementary cod liver oil, Adams and McCollum (1) concluded that neither the degree of healing as indicated by the line test, nor the change in ion product values (Ca and $PO_4$ in blood serum) toward normal, is proportional to the cod liver oil intake; that bone analyses lend no very definite information relative to the antirachitic potency of the oils under investigation; and that not only is the information indefinite but in addition its reliability is to be questioned, since such diverse values are obtained even under identical experimental conditions, that averages are, in their opinion, of doubtful significance.

In rats on a basal diet adequate except for vitamin D, Leigh-

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Clare and Soames (2) found growth and the ash content of bones roughly proportional to the potency and quantity of the supplementary cod liver oil. Although they suggest definite standards for a comparison of values, they feel that the degree of accuracy is small unless a very large number of animals is used.

Previous to the appearance of the papers just mentioned, we had already engaged in studying quantitatively the effect of graded allowances of vitamin D upon growth and calcification in young rats receiving a basal diet adequate in other respects, but decidedly deficient in vitamin D. In this work we have profited by the experience of several previous investigators, but in the interest of brevity we are obliged to forego further citations of literature.

We have found that a diet consisting of extracted casein (3), 18 per cent; Osborne and Mendel (4) salt mixture, 4 per cent; dry brewers' yeast, 10 per cent; sodium chloride, 1 per cent; dried spinach, 1 per cent; and corn-starch, 66 per cent, is adequate for rats under our experimental conditions, except with respect to vitamin D. It contains sufficient vitamin A to permit our rats to make approximately normal growth throughout the experimental period and to store the normal percentage of calcium in their bodies for their age and the calcium content of the diet (5) when vitamin D is supplied abundantly.

In the experiments here reported we used young white rats reared by mothers on a diet consisting of finely ground whole wheat, 2 parts; whole milk powder, 1 part; sodium chloride, 2 per cent of the weight of the wheat; with or without the addition of fresh lean beef (60 gm. per week) to the diet of the adults. These young, separated from their mothers when 28 days old, are known to have a considerable bodily store of vitamin D. Naturally this bodily store is smaller if they are separated when 21 days old

From 21 or 28 days of age until the close of the experimental period we fed the animals the basal vitamin D-deficient diet. During the experimental periods we continued some on the basal diet only (negative controls), while to others, matched in sex and weight, we supplied graded allowances of vitamin D (as carried by whole milk powder), or an abundance of vitamin D from a food source or through daily irradiation of the rats with ultra-violet

light (positive controls).   The experimental periods included the 28th to 56th days, the 52nd to 80th days, or the 110th to 166th days of life, respectively, in three series of rats separated from mothers when 28 days old; and the 21st to 56th days or the 52nd to 80th days, respectively, in two series separated when 21 days old.

The gain in weight was determined weekly; at the end of the experimental period, the femurs were dissected out, ashed, and analyzed for calcium according to the method of McCrudden as modified by Sherman and MacLeod (6).

TABLE I

*Comparison of Accuracy of Several Criteria for Expressing Degree of Calcification of Bones*

| | No. of cases | Fresh femur | | Dried extracted femur | | Ratio of ash to organic residue |
| | | Ash | Ca | Ash | Ca | |
| | | *per cent* | *per cent* | *per cent* | *per cent* | |
| Positive controls | 14 | 28 1±0 36 | 10 45±0 14 | 59 4±0 34 | 22 1±0 11 | 1 47±0 019 |
| Negative controls | 20 | 22 5±0 27 | 8 31±0 10 | 53 6±0 43 | 19 8±0 15 | 1 17±0 020 |
| Difference | | 5 6±0 45 | 2 14±0 17 | 5 8±0 55 | 2 3±0 19 | 0 30±0 028 |
| Ratio of difference to its probable error (critical ratio) | | 12 | 13 | 11 | 12 | 11 |

To express the degree of calcification in bones, different investigators have used as criteria one or more of the following: percentage of ash, or of calcium, in the fresh bone; percentage of ash, or of calcium, in the dried (alcohol-ether) extracted bone; or the ratio of the ash to the organic residue of the dried extracted bone.   In the course of these experiments we accumulated data by the use of which the degree of calcification (ossification) was expressed in each of these several terms for each of almost 300 rats.   In Table I are recorded the results obtained from the positive and negative controls of one series.   The corresponding data of all of our other experiments are concordant with these.

From the data of Table I, it appears that the order of accuracy is the same for each of the suggested criteria. The use of the percentage of ash, or of calcium in the fresh bone is recommended. The magnitude of the "critical ratio" indicates that certainly at one or two points midway between the two extremes, true differences in calcification resulting from grading the vitamin D intake can be measured.

In Tables II and III are recorded for two of our series the average gain in weight during the experimental periods and the percentage of calcium in the femurs resulting from the adminis-

TABLE II

*Effect upon Growth and Calcification of Adding Graded Portions of Whole Milk Powder between 21st and 56th Days*

| Additions of milk | No of cases | Average weight | | Average gain | Average weight | | Ca in fresh femur, per cent ± σ d | Improvement over negative controls | |
|---|---|---|---|---|---|---|---|---|---|
| | | Initial | Final | | Fresh femur | Femur Ca | | Per cent improvement | Relative to positive controls |
| | | gm | gm | gm | gm | gm | | | |
| Negative controls* | 13 | 30 | 77 | 47 | 0 304 | 0 0246 | 8 09±0 83 | | |
| 750 mg per wk | 13 | 31 | 92 | 61 | 0 347 | 0 0286 | 8 24±1 06 | 0 15 | 6 |
| 1500 " " " | 13 | 30 | 103 | 73 | 0 345 | 0 0311 | 9 01±0 76 | 0 92 | 39 |
| 2250 " " | 13 | 29 | 98 | 67 | 0 355 | 0 0353 | 9 94±0 73 | 1 85 | 78 |
| Positive controls* | 17 | 30 | 98 | 68 | 0 364 | 0 0381 | 10 47±0 60 | 2 38 | 100 |

* Includes some animals apparently deficient in antineuritic vitamin

tration of graded allowances of a vitamin D-containing food (whole milk powder). Concordant results were obtained in parallel experiments with graded allowances of fresh whole milk. Deposition of calcium in the femurs is markedly affected by supplementary vitamin D both between the time of separation of young from mothers and their 56th day of age, and between the 52nd and 80th days of age. Although we have encountered wide seasonal and litter variations in the level of calcification found in both negative and positive controls, we find that this does not interfere with our interpretation of results when we use as a basis

of comparison the improvement in test animals over their respective negative controls, relative to the improvement of the positive over the negative controls.

Practically normal calcification resulted when the basal diet was supplemented between the 52nd and 80th days of life with the vitamin D carried by whole (summer) milk powder, such as that used here, equivalent to 6 to 7 per cent of the dry weight of the diet (4.50 gm. of whole milk powder to 68 gm. of basal diet weekly), or 8 to 9 per cent of the calories. Similar results were obtained for animals on a diet adequate in other respects but practically

TABLE III.

*Effect upon Growth and Calcification of Adding Graded Portions of Whole Milk Powder between 52nd and 80th Days in Rats Separated When 21 Days Old.*

| ...s of milk | No. of cases | Average weight when: | | | Average gain, 52nd to 80th | Average weight. | | Ca in fresh femur, per cent ± a d. | Improvement over negative controls. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 21 days old. | 52 days old. | 80 days old. | Average gain, days. | Fresh femur. | Femur Ca. | | Per cent improvement. | Relative to positive controls. |
| | | *qm.* | *qm.* | *gm.* | *gm* | *gm* | *gm* | | | |
| controls | 13 | 24 | 71 | 94 | 23 | 0 346 | 0 0257 | 7.43±0 78 | | |
| er wk | 10 | 26 | 75 | 113 | 38 | 0 392 | 0 0348 | 8 88±0 37 | 1.45 | 39 |
| 3000 " " | 10 | 27 | 75 | 123 | 48 | 0 408 | 0 0413 | 10.12±0 73 | 2 69 | 72 |
| 4500 " " | 9 | 26 | 76 | 125 | 49 | 0 408 | 0.0439 | 10 76±0 77 | 3 33 | 87 |
| ntrols | 10 | 24 | 76 | 139 | 63 | 0 480 | 0 0536 | 11.18±0 49 | 3 75 | 100 |

free from vitamin D, when the diet was supplemented between their 21st and 56th days with somewhat more than 4 per cent of this whole (summer) milk powder (2.25 gm. of milk powder to 58 gm. of basal diet), 5 per cent of the calories. The addition of smaller graded portions of milk resulted in corresponding improvements in calcification over the negative controls.

The deposition of calcium in the femurs appears to be more closely proportional to the supplementary vitamin D furnished, than does gain in weight. The improvement in growth due to additional vitamin D-containing material between the 21st and

56th days is too small to encourage attempts to make use of **growth**
during that period as a quantitative measure of the vitamin **D**
furnished. However, as can readily be seen from Fig. 1, **after**
attenuation of the bodily store of the vitamin by feeding **a**
vitamin D-deficient diet for a month, grading the allowance **of**
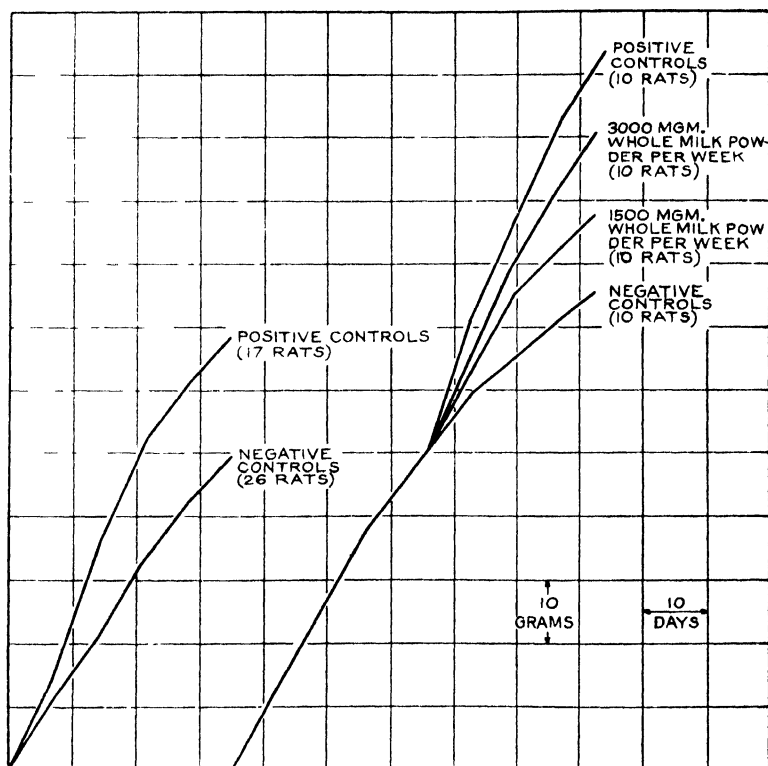


FIG 1. Effect upon growth of rats separated from mothers at 21 days
of age, of the addition of vitamin D between the 21st and 56th days, or be-
tween the 52nd and 80th days.

vitamin D is reflected in the gain in weight. We have been un-
successful in depleting our rats of their bodily stores of vitamin **D**
to the point that their growth is completely suspended, so that **we**
have been unable to perform satisfactory experiments using **limited**

growth as a quantitative criterion of the vitamin D content of food or other material under investigation. Throughout a period of over 3 months, the negative controls of one series continued to gain, on the average, about 6 gm. weekly. Incidentally, this is additional evidence (a) that the test animals had considerable bodily stores of vitamin D, which in the cases of families which had been for many generations on milk and wheat mixtures, must have come from the milk consumed by themselves and their mothers, and (b) that experiments on vitamin A conducted under the conditions employed in our laboratory, were not vitiated by shortage of vitamin D even before the recognition of this latter factor.

### SUMMARY AND CONCLUSIONS.

In young rats reared by mothers on a diet consisting largely of $\frac{2}{3}$ ground whole wheat, and $\frac{1}{3}$ whole milk powder, and transferred at the 21st or 28th day of age to a diet decidedly deficient in vitamin D, but adequate in other respects, practically normal calcification resulted by the 56th day of age in cases in which the basal diet had been supplemented by somewhat more than 5 per cent of the calories from whole (summer) milk powder, and by the 80th day of age in cases in which the basal diet had been supplemented during the preceding 4 weeks by the same milk powder to the extent of 8 to 9 per cent of the calories. Smaller graded portions of milk produced corresponding improvements in calcification over their respective negative controls.

The large numbers of experiments here briefly reported afford extensive and convincing evidence in confirmation of the fact that cow's milk as ordinarily produced in this country contains important amounts of vitamin D.

Under the conditions of these experiments as consistent responses in calcification were obtained when the experimental period followed immediately upon separation of the young from their mothers as when it was preceded by prolonged feeding of the vitamin D-deficient diet. This procedure insures vigorous animals, and permits the 4 or 5 week experimental period to be terminated at an early age, thus making use of the period of most rapid deposition of calcium, as well as reducing the time and expense involved in experimental work.

The percentage of calcium in the fresh femur is proportional to the supplementary vitamin D furnished within a sufficient range of values to permit of reasonably quantitative comparisons, when sufficient numbers of well controlled experiments are performed.

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# THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

## IV. CONCERNING THE SO CALLED TUBERCLE BACILLI WAX. ANALYSIS OF THE PURIFIED WAX.*

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### INTRODUCTION.

The literature dealing with the chemistry of the tubercle bacillus contains many references to the presence of a large amount of a wax-like substance in the lipid fraction. The nature of this waxy material, its properties, and functions have been the subject of several studies. It seems evident from published data that the substance is very stable and is saponified with great difficulty, but after complete saponification it presumably yields higher alcohols and fatty acids.

Attention was first called to this wax by Aronson (1) and all subsequent investigators have corroborated Aronson's observation. Kresling (2) reported that the unsaponifiable matter isolated from tubercle bacilli fat consisted of some higher alcohol. Similar observations have been reported by Bulloch and Macleod (3), Dorset and Emery (4), Auclair and Paris (5), Fontes (6), Panzer (7), and by Kozniewski (8). The chemical composition and properties of this alcohol were studied by Tamura (9) who described it as a monohydric alcohol of the formula $C_{29}H_{56}O$ to which he gave the name mykol. Mykol was acid-fast and was

probably responsible for the acid fastness of the bacilli.   Two
higher alcohols which differed in composition and properties from
mykol have been described by Bürger (10).   Although these
products were not studied very completely, Bürger assigned the
formula $C_{19}H_{36}O$ to one substance and $C_{15}H_{28}O$ to the other one.
The work of Tamura has been accepted generally by subsequent
investigators and Goris (11) in his work states that he isolated
substances corresponding in properties to mykol.   Although
mykol is stated not to give any of the sterol color reactions, it is
referred to by Wells, Dewitt, and Long (12), as follows: "Mykol
may thus be considered an alcohol, of the general group of sterols,
of which cholesterol is the best known member."

In an earlier work on the separation of the lipoids of tubercle
bacilli (13), we encountered a large amount of a wax-like material
in the chloroform extract.   It represented about 46 per cent of
the total lipids and 11 per cent of the dried bacteria.   The crude
wax was easily soluble in chloroform, benzene, and toluene and it
dissolved in less than its own weight of ether, but it was practically
insoluble in alcohol, methyl alcohol, or acetone.   The substance
contained a small amount of phosphorus and nitrogen and on
combustion it left about 1 per cent of ash.

An analysis of crude wax is given by Lewkowitsch (14) and
from the data published by Goris (11) it is evident that the
material which he examined contained some ash together with
phosphorus and nitrogen.   The occurrence of the two latter
elements has been regarded as evidence of the presence of con-
taminating phosphatides or lecithin.

For the present investigation the crude wax was purified by
precipitation from ether and toluene by methyl alcohol.   This
treatment yielded a white amorphous powder which represented
about 78 per cent of the original material and which we designate
as "purified wax."   The more soluble portion of the crude wax
was obtained from the mother liquors and since it formed a
yellowish salve-like mass at room temperature it was called "soft
wax."   The wax fractions mentioned above have been analyzed
and the present report deals with the composition of the puri-
fied wax.

All of the lipoid fractions that we prepared were tested bio-
logically in Dr. Sabin's laboratory at The Rockefeller Institute

for Medical Research and in these experiments it was found that the purified wax elicited a reaction similar to that of the phosphatide (15). It has been shown, however, that the active principle in the phosphatide is associated with a new saturated liquid fatty acid called phthioic acid (16).

It was of more than passing interest, therefore, to find an acid similar to phthioic acid among the mixed fatty acid obtained from the purified wax and that this substance also caused the formation of artificial tubercular tissue.

The purified wax differed decidedly in composition and properties from usual waxes. The material melted with decomposition between 200–205°. On hydrolysis it yielded nearly 40 per cent of water-soluble substances among which we found glycerophosphoric acid, reducing sugars which gave pentose reactions, and some nitrogen-containing compound that could not be identified. The ether-soluble constituents on the other hand contained a large amount of unsaponifiable wax-like material and a small amount of fatty acids.

It is evident from the products that are obtained on hydrolyzing the purified wax that the substance is not a wax in the ordinary meaning of that term but that it represents a complex phosphatide containing a large proportion of carbohydrates combined in its molecule.

The fraction which we have designated as unsaponifiable matter, above, may be the substance that has been called wax by earlier investigators and it seems self-evident that an identical product must have been obtained by those who have employed prolonged extraction of the bacilli with the so called Aronson's mixture, since the latter consists of 1 per cent hydrochloric acid in alcohol. The nature of this "unsaponifiable wax" has not yet been elucidated but further data regarding its composition and properties will be published shortly.

Not only the tubercle bacilli wax but the mykol of Tamura have been described as being acid-fast and the wax has been regarded as the carrier of acid fastness of the organism (17). It is interesting in this connection to note that the purified wax described in this paper, although it yields on hydrolysis 56 per cent of the unsaponifiable wax, has been found to be non-acid-fast, while the unsaponifiable wax was strongly acid-fast. This result

seems hardly possible unless the acid fastness should be found to depend upon the presence of free hydroxyl groups in the substance here termed unsaponifiable wax.   We are indebted to Professor W. L. Kulp of the Department of Bacteriology, Yale University, and to Dr. M. C. Kahn of Cornell University Medical College for the determinations on acid-fastness.

<div align="center">EXPERIMENTAL.</div>

The crude wax was of light yellow color and it resembled beeswax in appearance but was more brittle.   The substance was readily soluble in ether, chloroform, benzene, and toluene, but it was practically insoluble in alcohol, methyl alcohol, and in cold acetone.   Heated in a capillary tube, it melted at 50–51°.   Qualitative reactions indicated the presence of phosphorus and nitrogen and on combustion it left 1.02 per cent of ash.

<div align="center">*Purification of the Crude Wax.*</div>

The wax which weighed 427 gm. had been obtained as described in a former paper (13).   It was dissolved in 1 liter of anhydrous ether[1] and yielded a faintly cloudy solution which could not be cleared by filtration.   The addition of 1 liter of cold methyl alcohol caused a heavy nearly white precipitate.   After cooling in ice water the precipitate was filtered on a Buchner funnel and washed with methyl alcohol.   The substance was again precipitated two times from 1.5 liters of ether by adding 1.5 liters of methyl alcohol.   The product formed a nearly white amorphous powder.   It was distinctly less soluble in ether than in the beginning.   The ethereal solutions were cloudy and it was impossible to obtain clear solutions either by filtration or by centrifuging. The amount of the insoluble matter was, however, very small and it was in such a fine colloidal condition that nothing settled out on standing.   The substance was more soluble in chloroform, but even this solution was faintly cloudy.

For further purification the substance was dissolved in 500 cc. of toluene and except for the presence of a few paper fibers the

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[1] Throughout all operations air was excluded as far as possible by the use of carbon dioxide   All solvents were saturated with this gas and all flasks, desiccators, etc , were filled with carbon dioxide before any substance was introduced into them.

solution appeared to be perfectly clear. After filtering, the substance was precipitated by adding 2 volumes of methyl alcohol. It was precipitated a second time from toluene by adding acetone. After the mixture had been cooled in ice water the precipitate was filtered off, washed with methyl alcohol, and dried in a vacuum desiccator.

The purified wax obtained after the operations described above formed a nearly white powder and the yield was about 78 per cent of the original material. When heated in a capillary tube the substance turned yellow between 180–190° and melted with decomposition between 200–205°.

Analysis of the purified wax: P 0.407, 0.41; N 0.77; ash, 1.39 per cent.

### Recovery of Wax Residue from Mother Liquors.

The mother liquors from the precipitations mentioned above were concentrated by distillation, a stream of carbon dioxide being passed through the liquid, and the dark brown residue was dried in a current of carbon dioxide. The material was dissolved in toluene, and the solution was filtered. The addition of 2 volumes of methyl alcohol caused the separation of an oily precipitate. The mixture was shaken and cooled in ice water when the precipitate solidified. It was filtered, washed with methyl alcohol, and dried in a vacuum desiccator. At room temperature the substance formed a light brown salve-like mass that weighed 82 gm. Hence this fraction will be designated as "soft wax."

The final mother liquor, after it had been concentrated and dried in a current of carbon dioxide, formed a brown oil that weighed 10 gm.

Analysis of the soft wax: The substance contained only a trace of nitrogen and about 0.08 per cent of phosphorus.

### Saponification of the Purified Wax.

While we were under the impression that we were dealing with an ordinary wax, a preliminary saponification was made by boiling the purified substance for 8 hours with alcoholic potassium hydroxide.[2] When the substance was treated in this manner, two striking phenomena were observed: (a) The solid white powder gradually decomposed, forming two separate constituents

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[2] The air was displaced by nitrogen during the saponification,

that were insoluble in the boiling alcoholic solution.    One of these
constituents formed a colorless oil that solidified to a hard white
wax-like cake when allowed to cool to room temperature.    (b)
The other portion remained as a solid, somewhat sticky mass
on the bottom of the flask.

The two constituents were separated mechanically by decanting
the hot alcoholic solution containing the oily substance in suspen-
sion    The residue in the flask was treated repeatedly with boiling
alcoholic potassium hydroxide, but it did not dissolve.    It was
also insoluble in ether, but it was easily soluble in water.

*Examination of Ether-Soluble Constituents after Saponification
of the Purified Wax.*

The hot alcoholic solution obtained by decantation contained
a large amount of an insoluble colorless oil.    To insure complete
saponification the mixture was again refluxed for 12 hours.    On
cooling, the oily material formed a hard white cake on the bottom
of the flask.    The alcoholic solution was decanted and the solid
wax washed several times with alcohol.

The alcoholic solution and washings were concentrated by
distillation to a small volume and then diluted with water which
caused a small amount of insoluble matter to separate.    The mix-
ture was extracted several times with ether and the ethereal
solution was washed with water.

The alkaline aqueous solution containing the water-soluble
soaps was united with the washings from the ethereal solution.

The ethereal extract mentioned above was used to dissolve the
solid wax-like cake that had separated on cooling the saponifica-
tion mixture.    The substance dissolved slowly in the ether on
being warmed gently.    The ethereal solution was filtered, con-
centrated by distillation, and the warm residue was dried in a
current of carbon dioxide.    The substance was again boiled for
3.5 hours with alcoholic potassium hydroxide, diluted with 1
liter of hot water, and extracted with hot benzene.    The benzene
solution was washed with water, dried with sodium sulfate,
filtered, concentrated by distillation to about 100 cc., and mixed
with 350 cc. of acetone.    A heavy amorphous precipitate was
produced which, after cooling in ice water, was filtered on a
Buchner funnel and washed with acetone.    The substance formed

a white powder and it had been precipitated almost completely since the mother liquor yielded a very small amount of material when evaporated to dryness. For further purification the substance was precipitated once more from benzene and twice from chloroform by adding 3 volumes of acetone. The product which represents what would usually be designated as unsaponifiable matter, was a snow-white powder, and it had no sharp melting point. When heated in a capillary tube it sintered at about 60° and formed a slightly opaque melt at 64°. The substance was readily soluble in chloroform and in benzene, but it was not very soluble in cold ether and the ethereal solution was faintly cloudy. In warm ether it dissolved more readily and the solutions were clear. The ethereal solutions gelatinized on cooling, forming a semitransparent jelly. On warming, the mixture liquified, forming a clear solution. A 3 per cent solution in warm ether set to such a solid on cooling that the flask could be inverted without breaking the gel.

On combustion the substance left about 4.2 per cent of a fused colorless ash consisting of potassium carbonate. It seemed evident therefore that the material was either a potassium salt of some higher fatty acid or that it was contaminated with soaps. But every effort to fractionate the substance into different constituents proved fruitless.

### Removal of Potassium from the Unsaponifiable Matter.

The total purified material which weighed about 27 gm. was dissolved in 500 cc. of warm ether. Dry hydrochloric acid was passed into this solution, when a white precipitate of potassium chloride separated. The precipitate was filtered off and the ethereal solution was washed with water until the hydrochloric acid was removed. The solution was filtered, concentrated by distillation to about 400 cc., and mixed with 300 cc. of acetone. On cooling in ice water a snow-white precipitate separated which was filtered off and washed with acetone. The substance was again twice precipitated from 300 cc. of ether by adding 200 cc. of acetone and cooling in ice water. It was finally twice precipitated from 200 cc. of ether by cooling in a freezing mixture of ice and salt. After the snow-white amorphous powder had been dried in a vacuum desiccator over sulfuric acid it weighed 24.5

gm. The substance separated from the solutions mentioned above in a manner characteristic of crystalline bodies but when examined under the microscope, it was found to be non-crystalline, consisting of small transparent globular particles that varied considerably in size. When heated in a capillary tube, the substance softened at 56° and melted at 57-58°. When 0.4897 gm. of the substance was burned in a platinum crucible, it left no weighable ash. For analysis the substance was dried at 61° *in vacuo* over phosphorus pentoxide but there was no loss in weight.

0 1654 gm  substance: 0 2014 gm  H₂O and  0 4960 gm  CO₂

0 1180  "  "  . 0 1441  "  "  "  0 3532 "  "

Found   C 81 78, 81 63   H 13 62, 13 66.

The analytical results indicate that the relation between carbon and oxygen is in the ratio of 23.5:1. But since the substance possesses acid properties and also probably two free hydroxyl groups, it is evident that these numbers would have to be multiplied by 4 to account for the simplest formula containing one carboxyl and two hydroxyl groups. The calculation leads to the unusual and improbable formula of $C_{94}H_{188}O_4$. Since the substance does not crystallize and since it does not yield any crystalline derivatives, it is impossible to present any convincing evidence concerning the molecular magnitude of this compound. Attempts to determine its molecular weight by the boiling point or freezing point methods have led to abnormal results. For instance in the boiling point method, with benzene or ether as solvents, a depression of the boiling point was observed rather than a rise, and in the freezing point method, with naphthalene or camphor as solvents, molecular weights varying from 1046 to 1824 were obtained.

Values obtained on titrating the alcoholic solution of the substance with 0.1 N potassium hydroxide, or by analyses of the potassium salt, the silver salt, and of the acetyl derivatives all agree with the formula $C_{94}H_{188}O_4$. Further work is in progress in an attempt to elucidate the nature of this interesting compound.

*Properties of So Called Unsaponifiable Matter.*

The ash-free substance was readily soluble in benzene, toluene, chloroform, ligroin, petroleum ether, and in ether. On cooling

these solutions in ice water the substance separated as a dense white amorphous powder. The addition of cold alcohol or acetone caused a similar appearing precipitate. It was quite soluble in boiling acetone but on cooling the substance separated almost completely. It was very slightly soluble in alcohol; 1 part required about 500 parts of boiling absolute alcohol. In methyl alcohol or in glacial acetic acid it was very slightly soluble. It was apparently saturated since a chloroformic solution showed no visible absorption of bromine. The chloroformic solution of the potassium salt decolorized bromine and at the same time a white precipitate separated which evidently consisted of potassium bromide. When an ethereal solution of the substance was allowed to stand for 16 hours in contact with a large excess of bromine, a small amount of halogen was absorbed, apparently through substitution. The reaction product contained about 2 per cent of bromine. In the Liebermann-Burchard reaction no coloration whatever was produced.

No effect could be observed on shaking the substance with cold concentrated sulfuric acid. When treated with hot concentrated sulfuric acid, it melted and turned yellowish brown.

When boiled with a large excess of acetic anhydride, about 1:200, the substance dissolved completely, but on cooling it separated as small oily drops that solidified to a white hard cake when cold. When acetylated in this manner the substance took up acetic acid corresponding to two acetyl groups. When acetylated by the method of Einhorn and Hollandt (18), the presence of only one acetyl group could be demonstrated.

From the method by which it is obtained the substance would ordinarily be classified as unsaponifiable matter. The formation of acetyl derivatives would indicate that it possesses properties of a higher alcohol but as has been shown above it also possesses distinct acid properties. It might be argued that it is a mixture of higher alcohols and higher fatty acids, which mixtures, as is well known, are separated with great difficulty. However, all attempts to separate the substance into such constituents have proved entirely unsuccessful. Attempts have been made to separate acid constituents by means of the potassium or silver salts and laborious fractionations have been carried out with the ash-free substance without resulting in any noteworthy change

in properties.  The substance is extremely stable.  It may be boiled for many hours with alcoholic potassium hydroxide or with sodium ethoxide without any decomposition or change being noticed

For the present we are undecided whether to classify the material as unsaponifiable matter, as a higher hydroxy fatty acid, or simply as tubercle bacilli wax.  The unique properties of the substance would seem to justify either or all of these classifications.

### Isolation of Fatty Acids Occurring as Water-Soluble Soaps after Saponification of the Purified Wax.

The alkaline aqueous solutions of the saponification mixture after extraction with ether and benzene were acidified with hydrochloric acid.  The fatty acids were extracted with ether, the ethereal solution was washed with water, filtered, and the ether was distilled.  The oily residue was dried in a current of carbon dioxide.  On cooling it formed a yellowish crystalline solid which obviously contained some oily material.  It was combined with other similar fractions and reserved for complete analysis, as will be described later.

### Examination of Water-Soluble Constituents after Saponification of the Purified Wax.

It has been mentioned earlier that a notable amount of material, insoluble in alcohol and in ether, remained at the bottom of the flask after saponifying the purified wax.  This material was easily soluble in water.  The aqueous solution was extracted with ether to remove a small amount of wax-like substance.  The solution gave a white precipitate on the addition of alcohol.  On boiling with Fehling's solution no reduction occurred, but after the solution had been boiled for a few minutes with dilute hydrochloric acid it gave a heavy reduction on boiling with Fehling's solution.  It is evident, therefore, that the alcohol-insoluble material represented a carbohydrate complex which gave reducing sugars on hydrolysis.  Long boiling with alcoholic potassium hydroxide would undoubtedly alter or decompose any sugars that might be present in the compound.  In order to obtain any evidence regarding the nature of the carbohydrates present in the purified wax, it would be necessary to employ some other method of hydrolysis.

*Hydrolysis of the Purified Wax by Means of Acids.*

The purified wax was not wetted by water and the substance could not be brought into a state of suspension by continued grinding with water in a mortar. After a sample had been treated in this manner the wax particles were filtered off and the filtrate was tested for phosphorus and for carbohydrates, but only the faintest traces of these components could be detected. Evidently both phosphorus and carbohydrate were present in the wax in some combination that was insoluble in water.

A sample of the purified wax was boiled under a reflux condenser with 4 per cent sulfuric acid for 12 hours. Except for a slight yellow coloration of the powder that floated on top of the boiling acid, there was no evidence of any decomposition. The mixture was next heated in an autoclave to 120° for 2 hours. During this process the wax turned quite brown and it agglutinated to a hard, compact mass that apparently had not been hydrolyzed. The aqueous acid solution was faintly yellowish in color, but it contained only very slight traces of phosphorus or reducing sugars.

2 gm. of the wax were refluxed with 200 cc. of alcohol containing 5 per cent of sulfuric acid. After the mixture had been boiled for 1 hour evidence of hydrolysis could be observed and as the boiling was continued the solid particles of the wax were gradually converted into a colorless oil. After refluxing for 12 hours, all of the solid particles had disappeared. The mixture was diluted with water and extracted with ether. The ethereal solution was washed with water, filtered, and the ether was distilled. The residue after drying in a current of carbon dioxide weighed 1.4 gm. or 70 per cent of the wax. It was a faintly yellow oil when warm and a hard wax-like mass when cold.

A larger quantity of the wax was hydrolyzed in the manner just described and a special effort was made to determine the nature of the water-soluble constituents. The task proved very difficult, owing to the formation of large quantities of ethereal sulfates during the hydrolysis. Prolonged boiling of the aqueous solution was necessary in order to hydrolyze the ethereal sulfates, during which boiling perceptible decomposition of the carbohydrates occurred. As a result of these experiments it may be stated that the aqueous solution contained reducing sugars which, calculated as glucose, corresponded to 4.5 per cent of the wax.

Among the other water-soluble constituents, we were able to identify glycerophosphoric acid by means of the barium salt.

All of the nitrogen contained in the wax was present in the aqueous solution. Only a very small amount of the nitrogen could be accounted for as ammonia and no derivatives of choline could be isolated. In fact we were unable to isolate any nitrogen compound in solid form.

### Hydrolysis of the Purified Wax by Means of Alcohol and Hydrochloric Acid.

In order to avoid the troublesome ethereal sulfates which interfered with the determination of the water-soluble constituents we tried to employ alcohol containing 2 per cent of hydrochloric acid. This method worked very well and hydrolysis was complete after refluxing for 7 hours. The solid wax particles had then disappeared completely and the faintly brownish alcoholic solution contained a considerable amount of a colorless oil which solidified on cooling.

The alcoholic solution was concentrated to about one-half its volume, diluted with water, and extracted with ether. The ethereal solution was washed with water, filtered, and the ether was distilled. The residue after drying in a current of carbon dioxide formed a slightly yellow oil when warm and a nearly white solid when cold.

The ether-soluble constituents were boiled for 3 hours with 5 per cent alcoholic potassium hydroxide in order to saponify any esters that had formed during the hydrolysis. The saponification mixture was diluted with hot water and extracted with hot benzene. The aqueous solutions were saved for the isolation of fatty acids. The benzene extract was washed with dilute alkali and with water, dried over sodium sulfate, filtered, and the benzene was distilled. The residue was dissolved in warm ether and freed from potassium by passing in an excess of dry hydrochloric acid. The potassium chloride and the excess of hydrochloric acid were removed by washing with water. The ethereal solution was dried with sodium sulfate, filtered, and the ether was distilled. The residue after drying in a current of carbon dioxide formed a light yellow, thick oil which solidified to a hard wax-like cake when cold. For purification it was precipitated three

times from ether by adding cold acetone, yielding a practically snow-white amorphous powder which in all its properties corresponded to the so called unsaponifiable wax obtained after saponifying the purified wax with alcoholic potassium hydroxide.

### Isolation of the Fatty Acids.

The fatty acids were isolated from the alkaline solution after extracting the unsaponifiable wax with benzene. The solution was acidified with hydrochloric acid and extracted with ether. The ethereal solution was washed with water, filtered, and the ether was distilled. The residue consisting of fatty acids was dried in a current of carbon dioxide It formed a light yellow crystalline mass which evidently contained some liquid fatty acids.

TABLE I

*Ether-Soluble Material after Hydrolysis*

| Method of hydrolysis | Sulfuric acid and alcohol | | Hydrochloric acid and alcohol | |
|---|---|---|---|---|
| Time of hydrolysis, *hrs* | 12 | 9 | 7 | 7 |
| Total ether-soluble material, *per cent* | 70 | | 70 | 71 |
| Unsaponifiable wax, *per cent* | | 56 | 56 | 56 2 |
| Total fatty acids, *per cent* | | 4 6 | 3 6 | 5 6 |

### Summary of Ether-Soluble Constituents.

The yields of ether-soluble material, including unsaponifiable wax and fatty acids, obtained after hydrolyzing the purified wax with acids and alcohol, are shown in Table I.

It is evident from the data given above that the so called unsaponifiable wax represents the principal ether-soluble compound and that the true fatty acids were present only in small amount. The figures also show that a loss of about 10 per cent occurred during the second saponification and this loss is too great to be accounted for by the mere saponification of esters. It is possible that some water-soluble material was lost on extracting the fatty acids with ether because the residual aqueous solution was not examined.

*Examinations of Water-Soluble Constituents*

The aqueous acid solution after extraction of the ether-soluble constituents was concentrated *in vacuo* until the alcohol was removed and it was then made up to a convenient volume.

The solution contained all of the nitrogen and phosphorus that originally was contained in the wax as indicated by the following figures.

|   | In wax per cent | In aqueous solution per cent |
|---|---|---|
| P | 0 49 | 0 41 |
| N | 0 77 | 0 75 |

Practically all of the phosphorus was present in organic combination, presumably as glycerophosphoric acid.

The solution also contained reducing sugars, but the amount varied somewhat, depending upon the method of hydrolysis, as shown in Table II

TABLE II

*Reducing Sugars after Hydrolysis of Wax*

| Method of hydrolysis | Sulfuric acid and alcohol | Hydrochloric acid and alcohol |
|---|---|---|
|   | per cent | per cent |
| Reducing sugar calculated as glucose | 4 49 | 6 95 |

When some of the solution was heated with orcinol or phloroglucinol and hydrochloric acid, the characteristic pentose color reaction was obtained.

When the sugar solution was treated with phenylhydrazine two osazones were obtained

The more soluble osazone after several crystallizations from hot water and from a mixture of acetone and water was obtained as fine yellow needles. It melted at 165–166°. This melting point corresponds to that of arabinosazone but the melting point of a mixture of the osazone mentioned above with pure arabinosazone showed a large depression. Consequently the sugar could not have been arabinose.

A second, less soluble osazone, was also obtained. It was recrystallized several times from alcohol from which solvent it

separated in fine yellow needles.    When heated in a capillary tube, it melted with decomposition at 210°.

Unfortunately we were unable to secure any evidence regarding the nature of the nitrogen-containing compound.   Only a small amount of the nitrogen could be accounted for as ammonia.

### Separation of Fatty Acids by Means of Lead Soaps.

The various fractions of the mixed fatty acids were united, giving 10.9 gm., and dissolved in alcohol, neutralized with potassium hydroxide, diluted with water, and precipitated with a slight excess of lead acetate.    The lead soaps were filtered off, washed with water, dried *in vacuo*, and extracted with ether.    The ether-soluble and the ether-insoluble lead soaps were decomposed separately in the usual manner by shaking with dilute hydrochloric acid.

### Solid Saturated Fatty Acids.

The saturated fatty acids obtained from the ether-insoluble lead soap weighed 4.3 gm.   The substance was crystallized from alcohol and separated into three fractions.

Fraction 1 was recrystallized four times from alcohol and was obtained in small snow-white plates that weighed 0.5 gm.   The acid melted at 83–84° and evidently represents a slightly impure specimen of cerotic acid.   When titrated with 0.1 N alcoholic potassium hydroxide, 0.2648 gm. of the acid required 6.68 cc. of 0.1 N KOH, which corresponds to a molecular weight of 396. For $C_{26}H_{52}O_2$, calculated molecular weight 396.

Fraction 2 melted at 55–56°, and Fraction 3 melted at 54–55°. These acids which probably represented a mixture of palmitic and stearic acid were united, dissolved in alcohol, and separated by means of magnesium acetate into three fractions.   Each fraction was recrystallized twice from alcohol and once from methyl alcohol, but this treatment had not caused any separation since each substance melted at 56°.   When titrated with 0.1 N alcoholic potassium hydroxide, 0.3512 gm. of acid required 12.70 cc. of 0.1 N KOH, and 0.3069 gm. of acid required 11.11 cc. of 0.1 N KOH, which corresponds to a molecular weight of 276 in each case.

It is probable, therefore, that the acid is a mixture of about

equal parts of palmitic and stearic acid.   Such a eutectic mixture melts at 57° and would have a molecular weight of 270.

## Liquid Fatty Acids.

The liquid fatty acid isolated from the ether-soluble lead soaps was obtained as a slightly yellow oil that weighed 5.0 gm.   The iodine number, determined by the Hanus method, was only 11.3 and the molecular weight as determined by titration was 362 These values would indicate that a large proportion of the substance was a liquid saturated fatty acid with a higher molecular weight than that of oleic acid.

The mixture of liquid fatty acids was reduced with hydrogen in the presence of platinum oxide (19) and the reduced acid separated by means of the lead soap-ether treatment as described under phthioic acid (16).

The ether-insoluble lead soap gave a small amount of crystalline fatty acid which after being crystallized from alcohol and recrystallized from acetone melted at 68–69°.   The substance was undoubtedly stearic acid and it is most probable, therefore, that the unsaturated acid present in the original mixture was oleic acid

## Isolation of a Liquid Saturated Fatty Acid Analogous to Phthioic Acid.

The ether-soluble lead soap was decomposed by shaking with dilute hydrochloric acid and the mixture was washed with water until the lead chloride and hydrochloric acid were removed.   The ethereal solution was treated with norit, filtered, and the ether was distilled.   The faintly yellow oil, after drying *in vacuo* over sulfuric acid, weighed 3.1 gm.   When cooled in ice water it solidified to a white crystalline mass and liquified at 18–18.5°. At ordinary room temperature the substance was an odorless oil. The acid was saturated because in chloroform solution it did not decolorize a solution of bromine.   It was miscible in all proportions with alcohol and other organic solvents.

*Molecular Weight.*—The substance was dissolved in neutral alcohol and titrated with 0.1 N alcoholic potassium hydroxide. 0.4643 gm. of acid required 12.14 cc. of 0.1 N KOH.   Molecular weight 382.   0.3508 gm. of acid required 9.19 cc. of 0.1 N KOH. Molecular weight 381.

*Rotation.*—0.9253 gm. of acid was dissolved in alcohol and made up to 10 cc. In a 1 dm. tube $\alpha = -0.141°$, hence $[\alpha]_D^{20} = -1.63°$. In the same manner 0.8210 gm. of acid gave a reading of $-0.136°$ and $[\alpha]_D^{20} = -1.65°$.

For analysis the substance was dried *in vacuo* at 61° over phosphorus pentoxide but there was practically no loss in weight.

0 1520 gm substance: 0.1763 gm. $H_2O$ and 0.4318 gm. $CO_2$.
0 1094 "      "   : 0 1265 "  "   "  0 3098 "   "
Found. C 77 47, 77 23; H 12 97, 12.93.

The acid is most likely not pure but the analysis and neutralization value indicate that it must possess a high molecular weight and that it must be analogous to the phthioic acid (16) obtained from the phosphatide. It is interesting to note that the acid is levorotatory and as has been mentioned earlier it gave a physiological reaction on injection that was identical with that given by the dextrorotatory phthioic acid.

In conclusion it is a pleasure to acknowledge the helpful cooperation of Dr. William Charles White, Hygienic Laboratory, Washington, D. C., Professor Treat B. Johnson and Professor W. L. Kulp of Yale University, Dr. F. R. Sabin and Dr. C. A. Doan of The Rockefeller Institute for Medical Research, Dr. M. C. Kahn of Cornell University Medical College, H. K. Mulford and Company, and Parke, Davis and Company.

## SUMMARY.

The so called wax from tubercle bacilli has been purified and analyzed. The purified wax was a white powder that melted at 200–205°. On hydrolysis this substance yielded 71 per cent of ether-soluble and nearly 40 per cent of water-soluble constituents.

The greater portion, equivalent to 56 per cent of the total wax . consisted of an amorphous snow-white powder that possessed both acid and alcoholic properties. For the present this substance may be designated unsaponifiable wax.

A smaller amount of true fatty acids was isolated and these acids on separation yielded cerotic acid, probably a eutectic mixture of palmitic and stearic acids, oleic acid, and a liquid saturated fatty acid analogous to phthioic acid.

The water-soluble constituents consisted of glycerophosphoric acid, a mixture of reducing sugars that gave pentose reactions and some nitrogen-containing compound that could not be identified.

The purified wax that has been analyzed is accordingly not a wax but it represents essentially a complex phosphatide containing a large amount of carbohydrate.

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# THE SEPARATION OF CYSTINE FROM HISTIDINE: THE BASIC AMINO ACIDS OF HUMAN HAIR.*

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In the course of the precipitation of the silver compounds of arginine and histidine from a solution of the products of hydrolysis of proteins we have, in certain cases, observed the formation of an amino acid silver compound which is insoluble at reactions even more acid than pH 3.0. An examination of such a precipitate obtained during an investigation of zein revealed the presence in it of cystine. In the case of most of the proteins we have studied, the precipitation of histidine silver does not begin until the reaction has been carried well beyond pH 4.0, although when histidine is present in unusually high proportion, as in hemoglobin, histidine silver may begin to separate at somewhat more acid reactions. In view of the· observation mentioned above experiments were carried out on solutions of cystine in a minimal amount of sulfuric acid, which showed that this amino acid is nearly completely precipitated as a silver compound at pH 6.0; consequently cystine may be expected to occur in the crude histidine fractions secured from proteins in the usual way.

So far as we have been able to find, no particular attention has been paid to this possibility although Hedin in the paper in which he described his discovery of histidine (1) mentioned that the crude histidine fraction, secured by silver precipitation, contained a substance that gave the lead-blackening test for sulfur. This observation, however, had little significance in the period

before Morner demonstrated the presence of cystine in proteins (2) and it has apparently since been overlooked.

Data are given below showing that cystine is precipitated more or less completely by every heavy metal reagent that is customarily employed to precipitate histidine. It is therefore probable that cystine has been present as an impurity in the final histidine fractions secured in most protein analyses. The former universal practice of calculating the histidine content of such fractions from a nitrogen determination is consequently obviously objectionable. The dinitronaphtholsulfonic acid that we (3) have used extensively for the determination of the histidine content of the final histidine fractions secured in protein analysis, apparently yields reliable results provided the fractions are not too heavily contaminated with other amino acids. Difficulties are encountered, however, when the nitrogenous impurities amount to more than 20 per cent of the total nitrogen of the fraction. In order to increase the purity of the histidine fraction secured from proteins it has therefore seemed worth while to devise a procedure by which cystine can be separated from histidine.

The copper compound of cystine is very insoluble and separates promptly and completely from solution. Histidine, on the other hand, does not form an insoluble compound with copper under the conditions we have employed. To separate these two amino acids, therefore, it suffices to boil the solution containing them with an excess of copper hydroxide; after cooling, cystine copper together with the excess of copper hydroxide may be filtered off. Copper is subsequently removed from the filtrate by hydrogen sulfide and the solution is then treated in the usual way for the determination of histidine.

Copper hydroxide was first employed as a precipitant for cystine by Embden and the copper compound of this amino acid is described in a paper (4) in which he reported the isolation of cystine from horn.[1] It has also been used by Harris (5) to precipitate cystine from the mixture of amino acids removed from a protein hydrolysate by mercuric sulfate in 5 per cent sulfuric acid.

When cystine is boiled with strong acids, a change in certain of its properties occurs. The optical rotation of the solution

[1] Embden's discovery of cystine in horn was made independently of Morner (2) but his paper appeared somewhat later.

slowly diminishes, ultimately to zero; the product which is recovered after this treatment is more soluble than ordinary plate cystine and crystallizes in needles; analysis, however, indicates that it has the composition and many of the properties of cystine. This change has been studied by Neuberg and Meyer (6), by Hoffman and Gortner (7), and by others. There are 2 optically active carbon atoms in cystine and the formation of an inactive meso compound is therefore possible. The feebly levorotatory or inactive material secured after acid treatment may contain d-, l-, and mesocystine as well as a possible dl- compound. Andrews and de Beer (8) have investigated the solubility of partially and almost wholly racemized cystine preparations, with results indicating that several different substances were present in them. Our own investigations[2] of the phenylhydantoins prepared from similar material support this conclusion.

The cystine encountered in the histidine fraction in the course of an analysis of the basic amino acids of proteins is largely of this altered type and only low yields of poorly crystallized material can be secured from the precipitate obtained with copper hydroxide. In view of this the precipitation of cystine by copper hydroxide has been investigated both with preparations of pure plate cystine ($[\alpha]_D^{20} = -215°$) and partially racemized "needle cystine" ($[\alpha]_D^{20} = -41°$). Both compounds were nearly completely precipitated under the conditions employed. Similarly complete precipitations of the colorimetrically indicated cystine in histidine fractions derived from protein were also secured. The alteration in the physical properties of cystine brought about by the action of boiling acid does not, therefore, seriously affect the low solubility of the copper salt.

*Histidine Is Not Precipitated by Copper Hydroxide.*—Free histidine (0.500 gm.) was dissolved in 250 cc. of water containing 3 drops of dilute sulfuric acid, boiled ½ hour with 3 gm. of dry copper hydroxide, and then cooled ½ hour and filtered. The dissolved copper was removed by hydrogen sulfide. The solution contained 0.133 and 0.134 gm. of nitrogen (calculated 0.135 gm.) in two experiments, indicating that no histidine had been precipitated.

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[2] Unpublished observations.

*Cystine Is Nearly Completely Precipitated by Copper Hydroxide.*—
Pure plate cystine (0.500 gm.) was dissolved in 250 cc. of water
with the aid of a little dilute sulfuric acid. The solution was
boiled with 3 gm. of copper hydroxide' $\frac{1}{2}$ hour, allowed to stand $\frac{1}{2}$
hour, and filtered. After copper was removed, the filtrate was
analyzed for cystine by the Folin and Looney procedure, 0.0077
gm. or less than 2 per cent being found. Needle cystine ($[\alpha]_D^{20} =$
$-41°$) was likewise nearly completely precipitated.

*Cystine Can Be Separated from Histidine by Use of Copper Hy-*
*droxide.*—Mixtures of cystine (0.500 gm.) and free histidine (0.500
gm.) were dissolved in 250 cc. of water with the aid of sulfuric or
hydrochloric acid, were boiled with excess of copper hydroxide, and
filtered. Copper was removed from the filtrate and cystine was
determined colorimetrically in an aliquot part. Histidine was
recovered as dinitronaphtholsulfonate.

When the mixture was boiled 5 minutes with excess of copper
hydroxide and filtered at once, from 17 to 37 per cent of the cystine
escaped precipitation. When boiled $\frac{1}{2}$ hour with copper hydroxide
and filtered at once, about 8 per cent of the cystine was found in
the filtrate. When boiled $\frac{1}{2}$ hour and allowed to stand at least $\frac{1}{2}$
hour, less than 5 per cent of the cystine was found in the filtrate.
Thus in an experiment in which needle cystine was employed 0.024
gm. of cystine (4.8 per cent) and 0.426 gm. of histidine (85 per
cent) were found in the copper filtrate. The nature of the mineral
acid present did not affect the results.

*Precipitation of Cystine by Silver Salts.*—A hot saturated solution
of silver sulfate was added in excess to a solution of 2.0 gm. of
cystine in 400 cc. of water containing the minimal amount of sulfuric
acid required to dissolve the cystine (pH 1). A turbidity imme-
diately formed. To insure the elimination of chloride the solution
was centrifuged, whereby a trace of pale yellow flocculent precipi-
tate was removed. The supernatant fluid was turbid and precipi-
tate continued to deposit. Dilute sodium hydroxide was slowly
added with rapid mechanical stirring. The yellowish precipitate
increased in amount and became flocculent. Addition of alkali
was continued until the reaction reached pH 5.0 (colorimetric),
when the precipitate was removed. Silver was removed from the
filtrate by the addition of hydrochloric acid, the filtrate from the
silver chloride was concentrated, and cystine was determined by

the Folin and Looney method, 0.030 gm. or 1.5 per cent being found. In a similar experiment in which the reaction was carried to pH 6.0 only a trace of cystine remained in the filtrate. The silver compound of cystine secured under these conditions appears to be chiefly a double salt of the composition $(C_6H_{12}N_2S_2O_4Ag_2) \cdot Ag_2$-$SO_4$ and will be described more fully in a later communication.

*Precipitation of Cystine by Mercuric Sulfate in Acid Solution.*— Cystine (2.0 gm.) was dissolved in 250 cc. of 5 per cent sulfuric acid and 200 cc. of Hopkins' reagent were added. The white precipitate was removed after standing overnight, was washed with water, and decomposed by hydrogen sulfide. The filtrate from the mercuric sulfide contained 0.201 gm. of nitrogen, indicating that somewhat more than 86 per cent of the cystine had been precipitated.

*Precipitation of Cystine by Phosphotungstic Acid.*—1.0 gm. of cystine was dissolved in 100 cc. of 5 per cent sulfuric acid and treated with 20 cc. of 20 per cent phosphotungstic acid solution. The precipitate was removed after standing 24 hours and nitrogen was determined in the filtrate, 0.0204 gm. being found. This indicates that 82.5 per cent of the cystine was precipitated under these conditions.

### Basic Amino Acids of Human Hair.

Surprisingly little information is available in the literature concerning the amino acid composition of the keratins of animal hair. Although many studies of the cystine content of epidermal tissues have been made, Marston's recent examination of wool (9) is almost the only comprehensive investigation of one of them that has appeared. About 20 years ago Abderhalden and his students carried out a number of monoamino acid analyses of various keratoid tissues including wool (10) and horsehair (11), but few of these analyses were extended to the investigation of the basic amino acids. Argiris in 1907 (12) investigated the basic amino acids of horsehair, isolating 4.5 per cent of arginine and 1.1 per cent of lysine. He was unable to isolate histidine, although the nitrogen content of the histidine fraction indicated that approximately 0.6 per cent of this base was present. An examination of the few other published basic amino acid analyses of keratoid tissues shows that, except in Pregl's analysis of the membrane of *Scyllium stellare* (13), histidine was not actually isolated and that the indirectly determined proportions of this base are strikingly low.

Marston's determinations of the basic amino acids of wool were carried out by Van Slyke's indirect method, slightly modified on account of the high cystine content of the material. He obtained results indicating that wool yields 10.2 per cent of arginine, 6.9 per cent of histidine, and 2.8 per cent of lysine but these figures were reported with considerable reservation. Although a high proportion of arginine in wool is not surprising in view of the results we have secured upon human hair and horsehair by the direct method, the proportion of histidine reported by Marston is so unusually high as to suggest the desirability of a careful reexamination of wool by other methods. Hemoglobin is the only protein with which we are familiar that yields a larger proportion of this amino acid.

Our interest in the basic amino acid analysis of hair arose from the observation that cystine may find its way into histidine fractions secured by the silver precipitation method. It was felt that an investigation of the special analytical problems presented by the base analysis of a protein of high cystine and low histidine content might lead to a greater precision in the investigation of proteins of a more usual type. Human hair was selected because of its high cystine content, because a probable low histidine content was suggested by the recorded analysis of horsehair, as well as because of its physiological interest. The procedure employed was essentially that given in a previous paper (14). Only those modifications necessitated by the nature of the problem will receive more than passing attention.

The material, secured from a men's barber shop, was thoroughly extracted with gasoline, was washed free from odor with many changes of hot water, and then air-dried. It retained a small amount of fine sand. The hydrolysates were therefore filtered, the insoluble residue was ignited in a muffle, and the weight of the residual ash was subtracted from the weight of the sample taken. A further correction was applied for the moisture content of the material as used.

Hydrolysis was effected by boiling 255.4 gm. (corrected) of hair with 500 cc. of concentrated hydrochloric acid and 250 cc. of water for 8 hours. Preliminary experiments had shown that a maximal ratio of amino to total nitrogen was secured under these conditions. A small aliquot part was removed for analysis and the hydrolysate was concentrated several times to a sirup *in vacuo*.

The large proportion of cystine in hair necessitated a preliminary treatment of the hydrolysate with the object of removing as much of this amino acid as possible.  The sirup was therefore diluted, filtered, boiled with sufficient norit to remove most of the color, concentrated to 700 cc., and neutralized to brom-phenol blue (pH 4 to 5) with concentrated sodium hydroxide solution added slowly during rapid mechanical stirring.  An equal volume of alcohol was added and, after the mixture had stood 24 hours in the ice box, the precipitate of crude cystine was removed and dissolved in a minimal amount of hydrochloric acid.  The original hydrolysate contained cystine amounting to 16.5 per cent of the hair, the crude cystine precipitate contained 13.2 per cent, and the filtrate 3.0 per cent as determined by Folin and Looney's method.  From the crude cystine pure plate cystine amounting to 10.7 per cent of the hair was secured.   The specific rotation was $[\alpha]_D^{20} = -211.4°$[3] and the nitrogen content 11.54 per cent.  This result is similar to that of Rimington (16) who found that only about two-thirds of the colorimetrically indicated cystine derived from such tissues can be secured in the pure form.  The remaining cystine appears to be largely converted to a more soluble form and usually only a small part of it can be isolated as so called needle cystine.

The filtrate from the crude cystine was concentrated to remove alcohol, silver oxide[4] and sulfuric acid were added in excess, and then barium hydroxide until the reaction was at pH 7.0.   The precipitate containing silver compounds of cystine, histidine, etc., was decomposed with hydrochloric acid and the solution was freed from this acid by evaporation followed by precipitation with silver oxide at an acid reaction to brom-phenol blue.  A little cystine was doubtless precipitated at the same time.

Silver was removed from the filtrate by hydrogen sulfide, the solution was concentrated, and the histidine was precipitated by Hopkins' reagent in the usual way.   The mercuric sulfate precipitate was decomposed by hydrogen sulfide and, since the solution was found to contain cysteine, it was brought to pH 7.0 with barium hydroxide and aerated until the nitroprusside test was nega-

[3] Rotations were determined under the conditions advocated by Andrews (15); namely, 1 per cent solution in N hydrochloric acid.

[4] This procedure for adding silver sulfate was originally proposed by Kiesel (17).

tive.   The solution was then freed from barium and boiled with an excess of copper hydroxide for $\frac{1}{2}$ hour, allowed to stand until cold, and filtered.   Copper was removed from the filtrate which now gave a color with Folin and Looney's reagent, indicating the presence of only traces of cystine.   The histidine was then determined in the usual way by dinitronaphtholsulfonic acid.   When corrected for aliquot parts previously removed, the yield of disulfonate was 6.754 gm., equivalent to 1.337 gm. of histidine or 0.52 per cent of the hair; the sulfur content was 8.38 per cent, theory 8.17 per cent.   It should be noted that the histidine disulfonate secured from histidine fractions that have previously been purified by the treatment with copper separates in a much better crystallized and purer form than is usually the case.

The filtrate from the silver precipitation at pH 7.0 was faintly acidified with sulfuric acid, was concentrated, again treated with excess of silver, and the arginine silver was then precipitated with alkali.   To insure complete removal of arginine the filtrate (10 liters) was acidified with sulfuric acid, concentrated, and a second silver precipitate taken out at a volume of about 5 liters.   These precipitates were worked up and arginine equivalent to 5.98 per cent of the hair was isolated as dinitronaphtholsulfonate.

The filtrate from the arginine silver yielded lysine as picrate, equivalent to 2.41 per cent of the hair.

Difficulty was encountered in these operations owing to the presence of sodium which was particularly troublesome in the arginine fraction.   Moreover the short hydrolysis of this sample of hair gave rise to a possibility that all of the peptide linkages may not have been decomposed.   Another analysis was therefore performed on 222.4 gm. (corrected) of hair which had been hydrolyzed under the same conditions as before, except that boiling was continued for 17 hours.   This hydrolysate gave a slightly lower ratio of amino to total nitrogen (67.8 per cent as against 71.2 per cent) than the first and a slightly higher proportion of ammonia. This was to be expected owing to the instability of cystine in boiling concentrated acids.   Neutralization was effected by the use of basic lead acetate instead of sodium hydroxide.   Alcohol was not added after neutralization.   The lead acetate precipitate, when extracted with hot hydrochloric acid, ultimately yielded only 7.8 per cent of crude cystine.   Lead was removed from the filtrate by sulfuric

acid in the presence of 50 per cent alcohol and the last traces were precipitated as sulfide. Nearly all the hydrochloric acid had been removed along with the cystine as lead chloride. Arginine, histidine, and the remaining cystine were precipitated together as silver compounds at a strongly alkaline reaction in the usual way, the precipitate was decomposed by hydrogen sulfide, and histidine and cystine were thrown down as silver compounds at pH 7.2. This precipitate was decomposed by hydrochloric acid and reprecipitated at pH 7.3, to insure complete separation from the arginine. It was then decomposed by hydrochloric acid and the solution was concentrated several times to a sirup. Tests indicated the presence of considerable cystine and cysteine. The solution was therefore aerated at pH 7.0 as before, the barium was removed by the addition of a slight excess of sulfuric acid, and the solution was boiled with an excess of copper hydroxide and cooled. The copper precipitate was decomposed by hydrogen sulfide and found to contain 2.83 gm. of cystine. Of this 0.78 gm. was isolated as poorly developed needle-like crystals of nitrogen content 11.47 per cent and sulfur, 26.37 per cent; theory for cystine, 11.66 and 26.68 per cent respectively.

The filtrate from the copper hydroxide precipitate was freed from copper and from hydrochloric acid in the usual way; the histidine was precipitated by Hopkins' reagent and isolated as dinitronaphtholsulfonate, the equivalent of 0.36 per cent of the hair being found. The yield was lower than in the previous experiment, probably because of the more numerous operations with their attendant unavoidable losses.

The filtrates from the two silver precipitations at pH 7.2 were combined, acidified, concentrated, treated with an excess of silver, and the arginine therein was finally isolated as dinitronaphtholsulfonate. The amount found corresponded to 7.62 per cent of the hair; sulfur content 6.48 per cent, theory 6.56 per cent.

The filtrates from the precipitates of arginine silver were worked up for lysine. The crude lysine picrate, when recrystallized, gave two crops decomposing at 266° and 260° respectively, nitrogen content 18.73 per cent (theory 18.67 per cent), and weighing 13.37 gm. when corrected for aliquots previously removed. This is equivalent to 5.21 gm. of lysine or 2.34 per cent of the hair. The filtrates from the lysine picrate were carried through a second phos-

photungstic acid precipitation but the 3.01 gm. of crude picrate obtained melted at 218° and obviously contained arginine. It was therefore decomposed and subjected to a silver precipitation. It finally yielded arginine dinitronaphtholsulfonate of 6.62 per cent sulfur content, equivalent to 0.76 gm. of arginine (0.34 per cent of hair), and lysine picrate decomposing at 265°, equivalent to 0.27 gm. of lysine (0.12 per cent of the hair). These proportions were added to those previously secured and the final results of the two analyses are as follows:

*Basic Amino Acids in Human Hair.*

|  | Analysis I. per cent | Analysis II. per cent |
|---|---|---|
| Histidine | 0.52 | 0 36 |
| Arginine | 5.98 | 7.96 |
| Lysine | 2.41 | 2.46 |

The presence of arginine in the lysine fraction secured in the second analysis suggests that the strikingly high result for the proportion of arginine in human hair that was found may still be somewhat low. Our experience has shown that, on account of the quite appreciable solubility of arginine phosphotungstate, it is impossible to precipitate all of the arginine in such solutions by means of phosphotungstic acid. Furthermore there is little doubt that some arginine escaped isolation in the mother liquor of the crude mixed picrates. There is, therefore, every reason to believe that the arginine content of human hair may be well over 8 per cent.

That appreciable amounts of arginine can escape even the most carefully conducted silver precipitations further illustrates the difficulty of accurate base analyses of proteins and the necessity for more extensive investigation of the silver compounds of amino acids. We have mentioned in several previous papers that it is sometimes not easy to secure the test for the presence of excess of silver in amino acid solutions that are freely depositing silver sulfate crystals. This difficulty was occasionally encountered in the present work. While we have found that it can usually be surmounted by the liberal use of boiling silver sulfate solution, the explanation of the phenomenon is not yet clear. The behavior places a disconcerting check upon attempts to bring about complete precipitations of arginine by the use of concentrated solu-

tions and we recognize that it is a serious drawback to the use of silver oxide and sulfuric acid as a means of introducing large concentrations of silver ions. It is hoped that further study may furnish an explanation or an alternative procedure.

### Arginine Content of Horsehair.

In the course of some preliminary work arginine was isolated from horsehair. The material was secured in the form of packets of bristles prepared for brush making and was clean and uniform. Arginine to the extent of 7.3 per cent and 7.6 per cent was isolated in two experiments. These are minimal figures since they represent the results of a preparation rather than a determination and many of the precautions we have found necessary in quantitative work were omitted. The figures are given simply to illustrate the analogy between the high arginine content of two kinds of hair.

### SUMMARY.

In acid solution cystine forms a silver compound which becomes increasingly insoluble as the acidity of the solution is reduced. At pH 6.0 nearly all the cystine present in such a solution is precipitated. Since this amino acid is largely precipitated by the other heavy metal reagents customarily employed to throw down histidine, it inevitably finds its way into histidine fractions secured by the usual methods of protein analysis. Cystine copper is very insoluble and separates readily and completely when a solution of cystine is boiled with an excess of copper hydroxide and cooled. Under the same circumstances histidine remains in solution. Histidine fractions can therefore be freed from cystine by proper treatment with copper hydroxide, and this manipulation contributes materially to the ease and accuracy with which histidine can subsequently be determined as dinitronaphtholsulfonate in such fractions. As an illustration of the application of this procedure an analysis of the basic amino acids of human hair has been carried out which indicates that this tissue yields 0.5 per cent of histidine, 8.0 per cent of arginine, and 2.5 per cent of lysine. A colorimetric determination of cystine on the same sample indicated that 16.5 per cent of cystine was present.

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# AN IMPROVED FORM OF THE QUINHYDRONE ELECTRODE.

By GLENN E. CULLEN.

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During the last year we have been using an improved form of the simple micro quinhydrone electrode of Cullen and Biilman (1925) that increases both convenience and accuracy, especially when working with $CO_2$-containing solutions. The electrode, as can be seen from the diagram, Fig. 1, consists of two parts, the electrode and the electrode vessel. The modification consists of the addition to the electrode vessel of a side arm containing a glass plunger. The purpose of this side arm and plunger is to enable serum, or other $CO_2$-containing solutions, to be drawn up in the electrode vessel to entirely cover the platinum or gold electrode without disturbing the position of the electrode. In the older form as the glass rod carrying the electrode was withdrawn, the incoming fluid expanded into the space left vacant, and thus a fairly large surface of liquid was exposed to the air. With the new form the electrode proper remains in position, much less liquid is required, and exposure of the surface is almost elimininated.

The electrode vessel is easily made from an ordinary glass T-tube, about 7 mm. in diameter. A T-tube is selected in which the upper bar is perfectly straight, and in which there is no constriction at the joint, and one end of the upper bar is drawn out to a capillary. This capillary portion should be well centered, straight, and of fairly uniform bore. The glass of the capillary should be as thin as is consistent with strength, about 0.2 mm. The capillary portion is about 2 mm. in outside diameter, and about 3 to 5 cm. long, the only important requirement being that the end of the capillary must be at least 1 to $1\frac{1}{2}$ cm. below the end of the metal electrode. The tip of the capillary is slightly constricted. The

electrode proper, carrying the wire electrode, is prepared from glass tubing about 5 mm. in diameter that fits easily into the electrode vessel. A piece of c. p. platinum wire 0.60 mm. in diameter and about 3 cm. long is fused in a fine hydrogen-air flame to a slightly heavier piece of copper wire (No. 20) which serves as the lead-off
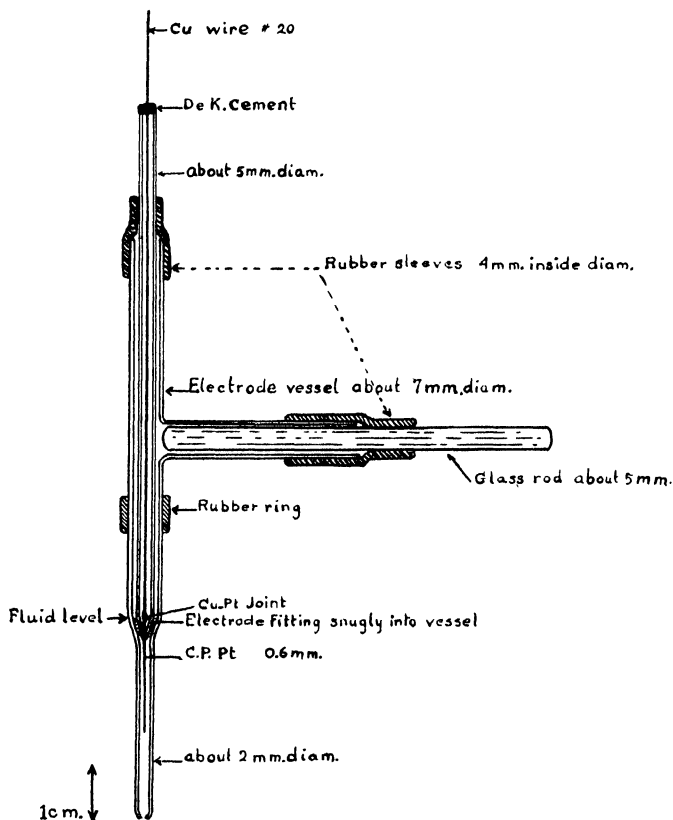


FIG. 1. Quinhydrone electrode and vessel assembled.

wire. This wire is then fused into the glass tube with the Cu-Pt joint just inside the tube. The glass is drawn down as a smooth tip over the wire to about the same shape as that of the funnel-shaped portion of the electrode vessel at the beginning electrode capillary portion so that when the electrode is in place only a capil-

lary space is left between the electrode and vessel. The hydrogen air flame is used for this operation. The upper end of the glass tubing is filled with De Khotinsky cement to keep fluids out.

A section of glass rod about 5 mm. in diameter that fits snugly but easily into the side arm is selected, and the ends rounded on a fine emery stone. The electrode and the plunger are held in position in the electrode vessel by short pieces of transparent acid-cured rubber tubing.

*Operation.*—The electrode vessel must be clean and dry. It is thoroughly rinsed out between successive determinations with distilled water, acid-free alcohol and ether, and then dried by suction. The inner surfaces of the rubber tubing sleeves are moistened with distilled water. The platinum wire is moistened with distilled water, touched to finely powdered quinhydrone so that a generous amount adheres, and the electrode, carrying its load of quinhydrone, is inserted into the electrode vessel as far as it will go. The glass plunger is inserted into the wet surface of the rubber tubing at the end of the side arm. The assembled apparatus is then dipped into the solution to be tested and by gentle withdrawal of the plunger the solution is drawn up into the capillary to cover the entire electrode. It is then placed at once in the KCl connecting vessel so that the surface of the fluid in the electrode is slightly above the level of the saturated KCl solution. This depth is easily adjusted by means of a small ring of rubber tubing about the electrode. The connecting vessel is either a 45 × 80 mm. weighing bottle or the base of a graduated cylinder, and it is fitted with a high melting paraffin stopper which contains openings for the saturated calomel electrode, for a thermometer, and for the quinhydrone electrode vessel.

*Precautions.*—The most serious source of error in this type of vessel is that of KCl diffusing up into the capillary. This is prevented by moistening the rubber joints, thus making them airtight. If no fluid leaks from the electrode after it has been filled, one may assume the joints are tight, but as added precaution, care is taken that the level of the fluid is always higher than in the KCl vessel. Every electrode is standardized against solutions of known pH, usually buffer mixtures of approximately the same pH as that of the solutions to be tested and the "*e*" of the whole system determined by use of the usual equation. $\mathrm{pH} = \dfrac{E-e}{KT}$.

In discussing the accuracy of the quinhydrone electrode, Cullen and Earle (1928) reported that only highest purity gold wire could be substituted for gold-plated platinum and suggested that impurities in the platinum wire might be the cause of the difficulties that they and others had experienced with bare platinum. This suggestion has been proved correct. The highest purity platinum wire, "c.p." of the American Platinum Company, gives identical results with 24 karat gold or with gold-plated platinum. This highest purity wire is now used exclusively in our electrodes. It has been found that it is desirable to purify the platinum ocsionally by heating to a dull red in the hydrogen flame. The platinum is contaminated or poisoned if one uses ordinary gas either in preparing the electrode or in purifying the platinum. An electrode which appears erratic is examined for roughness or for cracks with a low-powered binocular microscope, and immediately rejected if either is apparent.

*Advantages.*—The advantages of this modified form of electrode are several. First, because of simplicity and cheapness, it is easy to prepare and standardize a large number of these electrodes. The second advantage is that the use of thin capillary tubing makes it possible to attain temperature equilibrium in the KCl vessel almost immediately. Thirdly, the modificaton not only increases the convenience in handling the electrode, but makes it possible to use a very small quantity, 0.3 to 0.5 cc. Finally, and most important, it eliminates errors due to loss of $CO_2$ by making it possible to draw the solution up into the electrode entirely to cover the platinum still keeping the surface exposed negligible. The De Khotinsky cement used with the older form is no longer necessary. This is a great advantage, especially at 38° where this cement was not satisfactory.

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# STUDIES OF THE ACID-BASE CONDITION OF BLOOD.

## I. NORMAL VARIATION IN pH AND CARBON DIOXIDE CONTENT OF BLOOD SERA.

By I. P. EARLE* and GLENN E. CULLEN.

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The range of normal variations in the acid-base condition of the blood (pH and $CO_2$ content) has been studied by several investigators, including Cullen and Robinson (1), Myers and Booher (2), Bigwood (3), Marrack and Boone (4), and Koehler (5). Van Slyke (6) outlined the probable normal area and established the type of graph which seems the most suitable representation of acid-base variation. Austin and Cullen (7) added the $CO_2$ tension lines, used by Cullen and Jonas (8), to Van Slyke's graph and plotted the normal area of pH-total $CO_2$ values on the basis of all the data then known. As part of our class work here we have determined the values for pH and $CO_2$ content of the blood of students from the medical school classes. Earle and Cullen (9) reported last year that the values obtained in Nashville averaged a definitely higher pH than those obtained by Cullen and Robinson in Philadelphia, in 1923. The determinations reported last year were made in May under uncomfortably warm weather conditions and the question was raised as to whether or not this factor influenced the results. This study has been continued and the present report contains the values obtained with two more groups of students studied in March, 1928, and January, 1929.

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* Presented by I. P. Earle to the Graduate School of Vanderbilt University in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

*Experimental Technique.*

*Blood Sampling and Preparation of Serum.*—The blood samples were taken from an arm vein through a 19 or 20 gauge chromium steel needle into a centrifuge tube containing paraffin oil. The rubber and glass tubing which conduct the blood from the vein to the bottom of the centrifuge tube containing oil, must be of such small bore (2 to 3 mm.) that the blood advances in a solid column. The rubber tubing was carefully cleaned and dried, transparent, acid-cured tubing (A. H. Thomas No. 8840). Only Pyrex glass tubing was used. A tourniquet was used during the insertion of the needle but was released as soon as the blood began to flow. 15 to 20 cc. of blood were taken into a centrifuge tube under mineral oil. No anticoagulant was used. After all but a thin layer of the mineral oil was removed, the blood was immediately covered with melted paraffin, centrifuged for 15 to 20 minutes, and the serum removed at once with precautions to prevent loss of CO$_2$. Any serum showing hemolysis, which occurs rarely with this technique, was rejected for this study. In five cases it was not possible to draw blood without continued stasis. The values for these bloods are not included, but it should be remarked that instead of being uniformly more acid, two of these were around pH 7.50.

*Total CO$_2$ Content Determinations.*—These must be considered in two groups. Those made in 1928 were made on blood samples drawn usually by the students under our supervision. The serum was removed after centrifuging, and the pH was determined by one of us. The serum remaining was then given to the student who made the CO$_2$ determination with a Van Slyke constant pressure apparatus. These CO$_2$ determinations are accurate only to about 1 volume per cent. The student had previously been required to run CO$_2$ determinations on Na$_2$CO$_3$ solutions until he could get theoretical results within 1 per cent.

The results given for 1929 were all made on samples drawn by us. The CO$_2$ determinations were made on the serum by one of the staff, on the newest type Van Slyke constant volume closed manometric apparatus, using the Van Slyke-Sendroy (10) tables for calculating CO$_2$ content. They are accurate to within at least 0.5 volume per cent.

*pH Determinations.*—As soon as the serum was obtained after centrifuging the blood, duplicate determinations were made by

Cullen's colorimetric method with refinements in preparation of diluent, etc. (see Earle and Cullen (9)). The readings, corrected to 20°, were all calculated to 38° by subtracting 0.23. The readings were all made at 20–21° using the Daylight lamp made for us by Palo Company.

Most of the high pH values of the 1929 series were checked by the quinhydrone electrode with the technique developed by Cullen and Earle (11).      .

$CO_2$ *Tension Calculation.*—In calculating the $pCO_2$ lines of Fig. 1, representing the upper and lower limits of normal $CO_2$ tension, the new solubility coefficient of Van Slyke, Sendroy, Hastings, and Neill (12) and a pK′ value of 6.10 were used. (See discussion in the following paper (13).)

### Discussion of Results.

These new data are applied to a revision of the chart showing the normal area published by Austin and Cullen, Fig. 1. The summary chart also includes thirteen determinations taken before rising, from the study on diurnal variations reported in the following paper.

It is at once evident that the pH values of normal blood sera tend to run higher in all of our normals than has been found elsewhere. Since the present data were obtained in January and March, the weather influence suggested above is ruled out. It is also to be noted that our data raise the upper $CO_2$ absorption curve, *i.e.* the upper boundary of the so called normal area, by 2 volume per cent $CO_2$.

The data raise the upper limit of normal pH values slightly but the real significance of the results is that they fall predominantly between pH 7.4 and pH 7.5 rather than, as was our experience before, between pH 7.3 and pH 7.4. The explanation of this is not entirely clear. It is possible that 0.01 to 0.02 of the change may be due to refinement in technique. We now always use as diluent a saline indicator solution which has been freed of $CO_2$ by aeration. In the study made by Cullen and Robinson the saline was adjusted by addition of NaOH without removal of $CO_2$. Our experience and that of Hastings and Sendroy (14) indicate that removal of $CO_2$ tends to increase the pH reading 0.01 and perhaps 0.02 pH. This, however, accounts for only part of

the shift.    We feel that it is not due to any error in the colorimetric
correction factor, $C$, since we have checked most of the high values
with the quinhydrone electrode.    We found, in nearly all cases
where parallel determinations were made, the difference of 0.14 pH
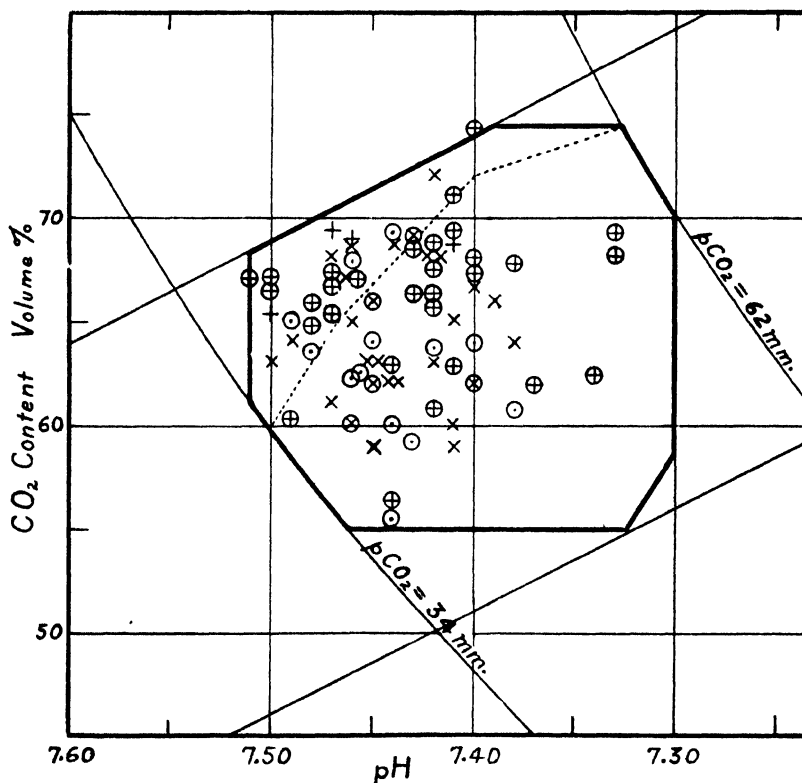


FIG. 1. Graph showing pH values in relation to normal limits of CO₂ tension ($p$CO₂ curve) and CO₂ content (CO₂ absorption curves) for human sera. Dotted line indicates "normal area," Austin and Cullen; solid line, normal area extended to include present data; × 1928 series, p.m.; ⊗ 1928 series, a.m.; + 1929 series, p.m.; ⊕ 1929 series, a.m.; ⊙ 1929 diurnal series, a.m., before rising.    The upper [CO₂] curve is 2 volume per cent higher than in the charts of Austin and Cullen's monograph.

between colorimetric and quinhydrone determinations ($pH_{20°}$ − $pH_{Q_{20}} = 0.14$) which has been previously reported (Cullen and Earle (11).    Incidentally, this increases our confidence in the

reliability of the quinhydrone electrode for determinations at room temperature.

Some of the blood samples were taken in the afternoon instead of in the morning. In the following paper we show that there is a large variation during the day, and that the tendency is toward a progressive increase in pH throughout the day. Most of the determinations on the 1928 group were taken in the afternoon. The blood for these determinations was always taken after 3.30 or 4 p.m. in order to minimize the effect of the noon meal.

If the pH values shown on the chart are grouped in two groups, those taken in the morning and those in the afternoon, it is found that 40 per cent of the bloods drawn in the morning are more alkaline than pH 7.45, and 60 per cent are less alkaline; while 48 per cent of the afternoon bloods are more alkaline than pH 7.45 with 52 per cent less alkaline. Although the total number of results is rather small for this type of analysis the difference in distribution between 40 per cent and 60 per cent as compared with 48 per cent and 52 per cent shows a definite tendency toward greater alkalinity in the afternoon. This point is discussed further in the following paper.

CONCLUSIONS.

The pH values obtained from the groups of people studied lie predominantly between 7.4 to 7.5, with an upper limit of 7.52. We feel that with these additional data the upper limit of the normal area must be considered to be somewhat higher than that given by Austin and Cullen. One question that at once arises is the interpretation of this increase in normal area in relation to previously reported conditions of abnormally high pH values. For example, some of the pH figures, which Bigwood (15) uses as evidence of increased alkalinity in epilepsy, fall within this area. However, the normal values obtained by Bigwood do not run as high as ours and his normal values must be used for the comparison with his pathological values, obtained under the same conditions. Therefore, the difference he has found should be accepted as satisfactory proof of a tendency toward increased alkalinity in epilepsy. The same argument holds true for the studies of Drucker and Faber (16) on infantile tetany. Their studies were made with capillary blood from the heel, with Hawkin's (17) modi-

fication of Cullen's method. With the technique developed for this method of taking blood, Drucker and Cullen (18) included a small correction for loss of CO$_2$ in oil so that the comparison must be made between the values for the pathological conditions and for normal controls made with the same technique.

## SUMMARY.

Determinations, made on normal individuals, of pH and CO$_2$ content of blood sera indicate that the zone of normal variations must be somewhat extended. The new data are presented and utilized in a revision of the graph showing normal values for pH and CO$_2$ content.

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# STUDIES OF THE ACID-BASE CONDITION OF BLOOD.

## II. PHYSIOLOGICAL CHANGES IN ACID-BASE CONDITION THROUGHOUT THE DAY.

By GLENN E. CULLEN AND I. P. EARLE.*

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The range of normal variation in the acid-base condition of the blood—pH and $CO_2$ content—has been studied by several investigators, and it is well established that there may be considerable fluctuation not only in different individuals, but in one individual from day to day. The influence of various physiological processes has also been investigated, such as exercise (Barr *et al.* (1)), of overventilation (Henderson and Haggard (2)), sleep (Kunze (3) and Endres (4)), and of digestion (Jansen and Karbaum (5)). However, we have been unable to find data showing the possible variation for an individual throughout the course of a day. In studying the variations in pH and $CO_2$ reported in the preceding paper, we attempted to correlate the results with the various activities of the individual but realized that we had no basic values for comparison.

The present study was planned to acquire information as to the extent and nature of the fluctuations in normal individuals throughout an ordinary day. The data presented here are sufficient to indicate that these fluctuations may be rather large and to suggest that some generally accepted opinions in regard to the influence of various conditions may need revision.

### EXPERIMENTAL.

*Plan of Study.*—It was desired first to determine if the variations in the general acid-base condition during an ordinary day were

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* Presented by I. P. Earle to the Graduate School of Vanderbilt University in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

large enough to be of significance, and if they were the same for different individuals. Therefore, no attempt was made to regulate the conduct of the individual other than to insure that it was not unusual and that the preceding day had included no unusual activities or significant changes in routine. None of the individuals had colds or headaches or had taken any drugs. The meals were not regulated except by habit, unless indicated in the protocols; the breakfasts were light and the noon and evening meals were substantial and approximately equal. Certain breaks in the routine; in one case, sleep in the evening, and in two cases, running to the laboratory, are noted on the charts.

The group studied includes both men and women and it should be reported that all of the women were studied between menstrual periods.

*Time of Taking Blood.*—The first sample for each day (except with Subjects 2, 6, and 7) was taken in the morning *before rising*, at the usual time of awakening and before the subject had made any movement beyond that necessary to put the arm in a convenient position for vein puncture. If the individual who was to be studied reported any unusual restlessness for the night the run was postponed. Following this, with the first three subjects studied, Experiments 1a, 3a, and 4, blood was taken again an hour or so after breakfast. There appeared to be so little effect from the breakfast that on subsequent days the after breakfast determination was omitted. This is to be regretted. After the noon and evening meals, samples were taken at different intervals in order to note the effect of different stages of digestion. The last sample was taken in all cases near 10 p.m. in order to get the determination finished that evening.

*Variables Studied.*—In all cases $CO_2$ content and pH of the serum were determined. Urine samples were obtained for pH determinations immediately following, and also at intervals between the drawing of blood samples. In the latter half of the series, total and titratable acidity determinations were also made on all urine samples. The urine studies are being continued and will be reported separately later. At present there seems to be no obvious correlation between urine pH and blood pH levels. From the $CO_2$ content and pH value, the $CO_2$ tension has been calculated for each blood sample and added to the charts showing pH and $[CO_2]$ determinations.

*Experimental Technique.*

*Blood Sampling and Preparation of Sera.*—The procedure outlined in the preceding paper (6) was followed. The present study involves repeated vein punctures and it should be noted that with the use of sharp needles, the discomfort due to successive vein punctures was so little as to eliminate pain disturbances. It was observed that the morning vein puncture with the subject comfortably relaxed required more care than the later ones.

This occasion may be taken to emphasize the fact that with the present universal use of venous puncture, often too little care is taken to insure properly sharpened needles. Needles that are not sharp enough or that have ragged edges disturb or pain the subject and the reaction to such disturbance may easily introduce changes in the vasomotor bed with corresponding changes in $CO_2$ tension and content. Properly sharpened needles slip into the vein so easily that there is no disturbance and the tissue damage is so slight that repeated punctures can be made in the same vein at the same site. In this laboratory the same care is used in the examination of needles that is used in examining platinum electrodes; *i.e.*, all needles are inspected with the low power binocular microscope.

*pH Determinations.*—The pH of each serum was determined at once in duplicate or triplicate by Cullen's colorimetric method (7) with the refinements reported by Earle and Cullen (8). All pH values are corrected to 38°. In addition to these determinations the pH of at least one serum sample of each day's experiment was checked by determination with the quinhydrone electrode with the technique developed by Cullen and Earle (9). On two days, both colorimetric and quinhydrone electrode determinations were made on every sample of serum and in several cases both methods were used on the samples taken before and after meals. Every pH determination that appeared unusual or that was difficult to read colorimetrically was repeated with the quinhydrone electrode. In all cases the two methods checked within ± 0.02 pH of the constant difference of 0.14 at 20°. There is a suggestion that the sera that have, during digestion, a high fat content, tend to give a higher correction by 0.01 or 0.02 pH than the clear sera obtained before meals. For example, the difference between the two methods for one individual was 0.13 pH before the meal and 0.15 pH for the fatty serum after the meal.

*CO₂ Content.*—The $CO_2$ values are for total $CO_2$ content of serum, designated as $CO_2$ and expressed in terms of volume per cent rather than as millimolar concentration in order to conform to the usual clinical usage. All determinations were made on 1 cc. samples of serum with Van Slyke's 50 cc. manometric apparatus with Van Slyke and Sendroy's calculation tables (10). The $CO_2$ determinations on the evening samples were usually made the following morning on sera which had been covered with a layer of paraffin, chilled at once, and kept in the refrigerator overnight.

*Calculation of CO₂ Tension.*—The $CO_2$ tension of the serum was calculated for 38° by the Henderson-Hasselbalch equation using a pK' value of 6.10 and the new value for $\alpha CO_2$ of 0.510, obtained by Van Slyke, Sendroy, Hastings, and Neill (11). These calculated values are recorded in mm. of mercury.

Van Slyke and Sendroy (12) used these values in constructing a most convenient nomogram and discussed the reasons for their use. There is no question that the new $\alpha CO_2$ should be used instead of the older Bohr value, but its use involves an adjustment of pK'. The pK' values obtained by Cullen, Keeler, and Robinson (13) and by Hastings, Sendroy, and Van Slyke (14) differ by about 0.04. The reason for this difference is so far unknown for the method and the technique used in both studies are all developed in Van Slyke's laboratory. Hastings, Sendroy, and Van Slyke also discuss this question. These discrepancies should be considered, if the present results were being compared with previous data, but in this study the interest is in changes in $CO_2$ tension rather than in absolute values. The $CO_2$ tension values calculated here are in agreement with those obtained by the use of the new Van Slyke-Sendroy nomogram.

### Results.

The data are presented in Figs. 1 to 4 with the values for the pH, $CO_2$ content, and $CO_2$ tension for each sample plotted against the time when the blood was drawn. The meals were eaten at the times indicated by the heavy arrows on the abscissæ. Dotted lines indicate that no values at the usual time were obtained. It is recognized that the curves formed by connecting the points representing the successive determinations often present a somewhat distorted picture of the rate of change, since the successive
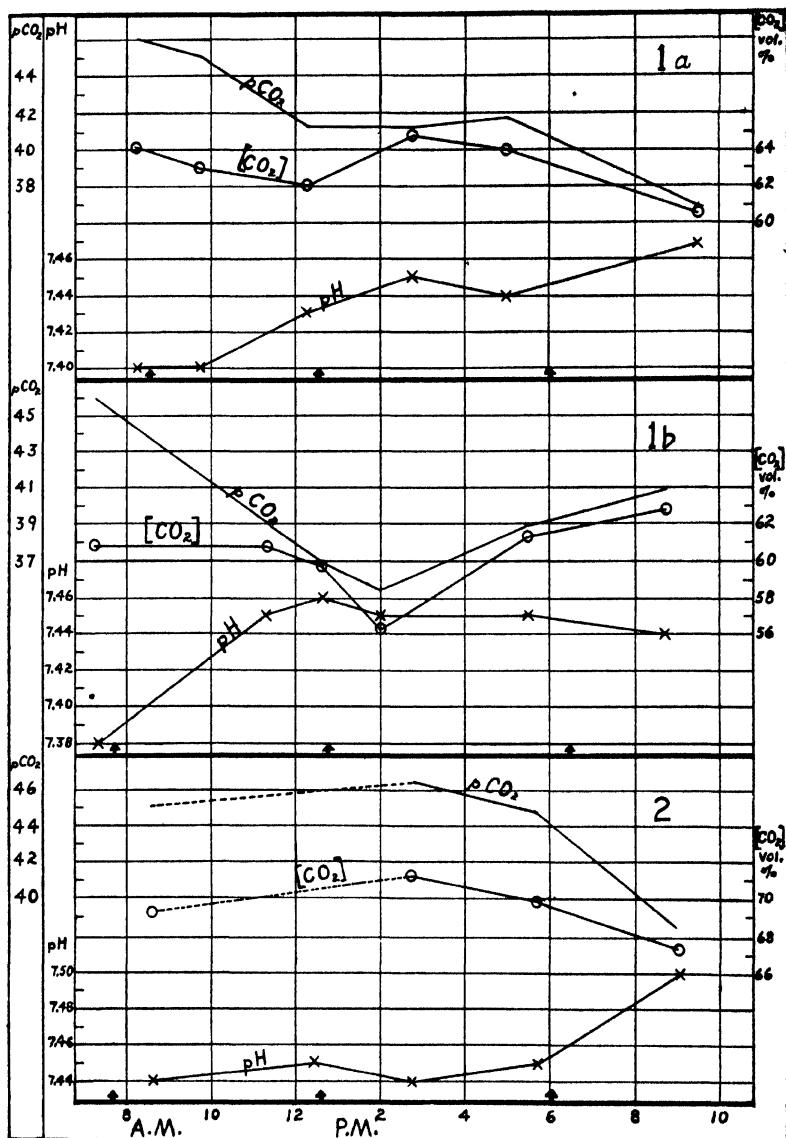
Fig. 1. Curves showing changes in acid-base condition with time. Numbers indicate subject; *a* and *b* different experiments on the same subject; arrows indicate meals.
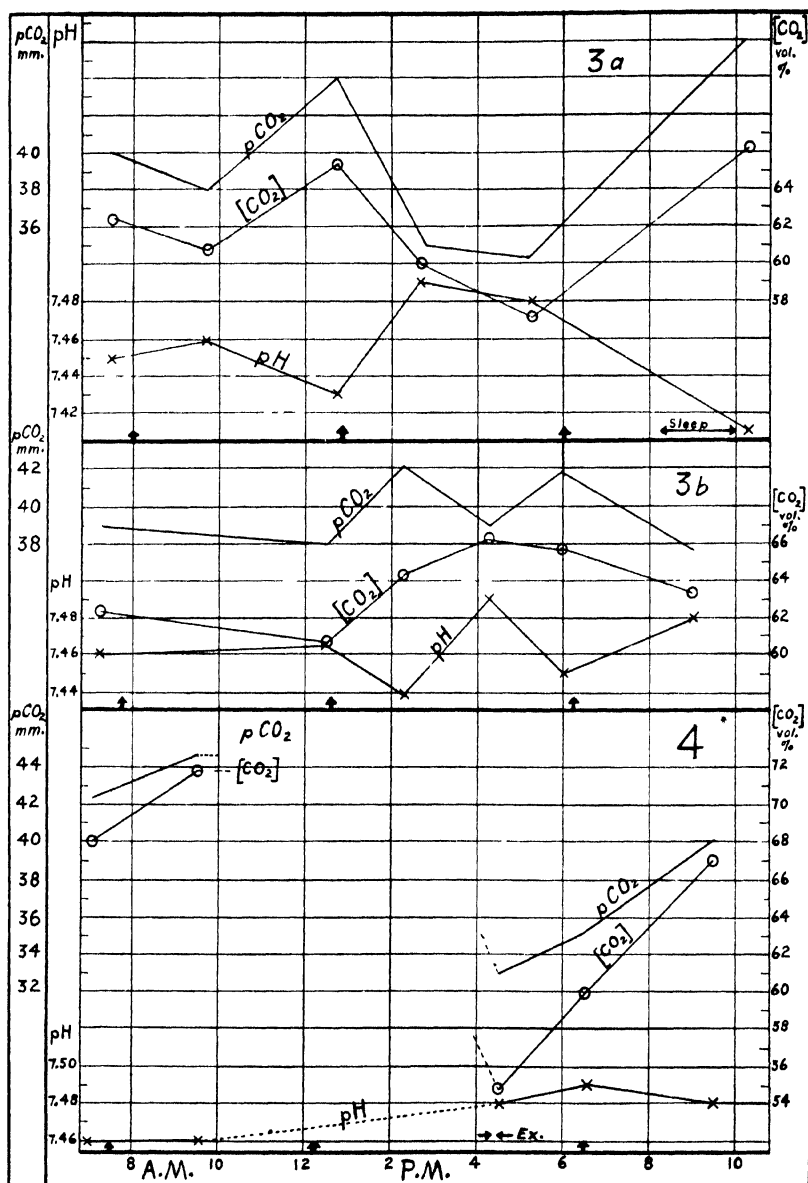
FIG. 2. See legend below FIG. 1.
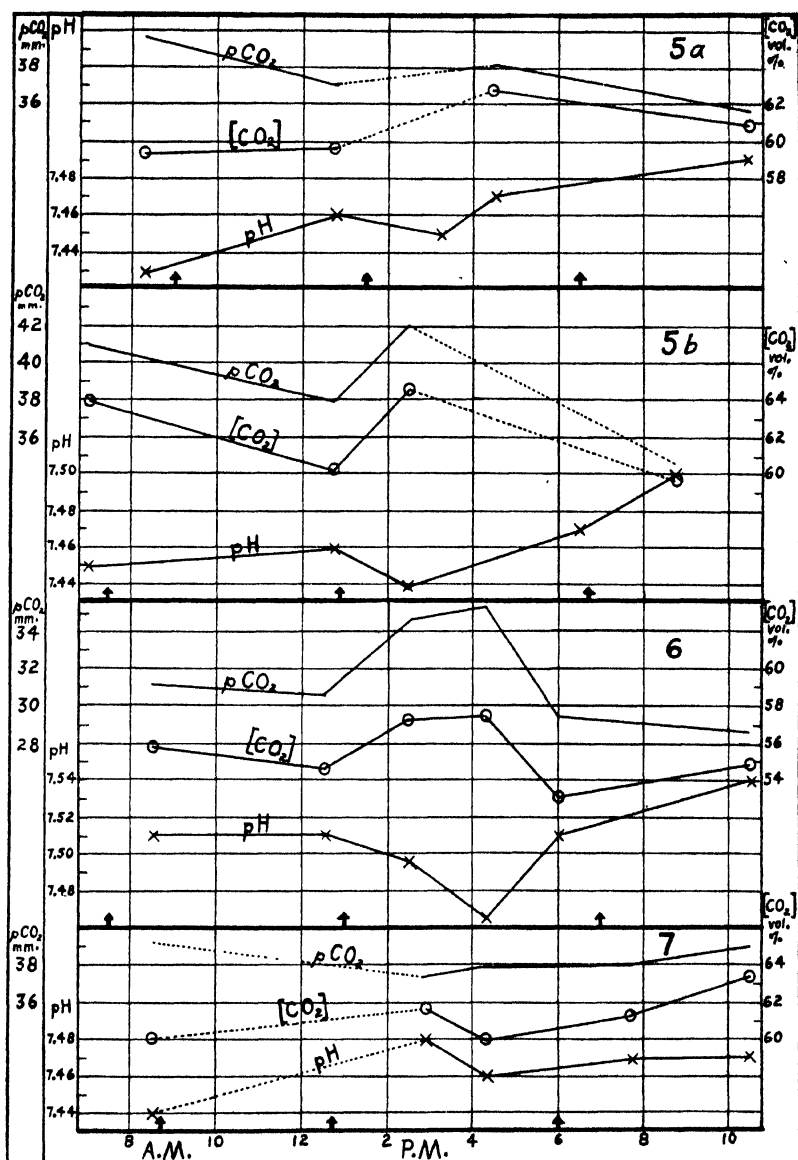
FIG. 3. See legend below Fig. 1.
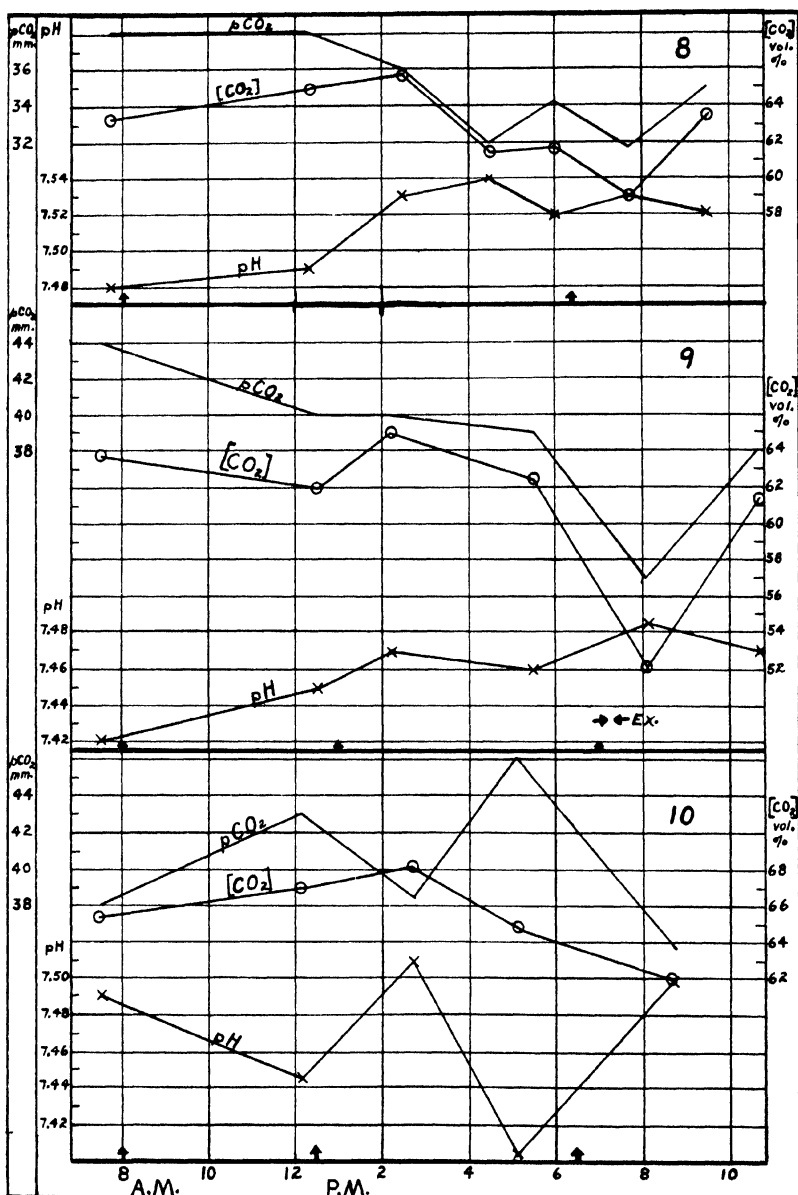
FIG. 4 Curves showing changes in acid-base condition with time.
Numbers indicate subjects; arrows indicate meals.

determinations were in many instances too far apart to indicate when the change began.

The charts are not arranged in chronological order but are so arranged that duplicate runs on three subjects may be easily compared.

This series includes thirteen studies on ten subjects, six men and four women. Two satisfactory separate runs were made on each of two women (Experiments 1a, 1b, 3a, and 3b). Two studies on one man (Experiments 5a and 5b) were not so satisfactory as he is hard to bleed and one sample of each run yielded too little serum for $CO_2$ determinations.

In each of two other studies, Subjects 7 and 4, one sample of each series was lost after centrifuging when it was too late to repeat the determinations. This was not serious in the case of Subject 7, before lunch, but is to be regretted in the case of Subject 4 which will be discussed below. Data concerning the subjects are given in the protocols below.

*Morning Periods.*—In three experiments, Nos. 1a, 2a, and 4, at the beginning of the series samples were obtained before rising and after breakfast. The changes in the second pH values as compared to the initial were 0 pH, $+0.01$ pH, and 0 pH. The corresponding $CO_2$ changes were $+1.0$ volume per cent, $-1.5$ volume per cent, and $+4$ volume per cent. It, therefore, appeared that the acid-base condition which had been attained by the end of the sleep period was fairly stable and that the early morning activity did not markedly change it. This conclusion seems to be supported by the fact that the changes that had occurred by noon, before lunch, in nine of the eleven available pH comparisons, were less than 0.03 pH, with corresponding $CO_2$ changes of 0 to 3.7, volume per cent. However, Subject 1 showed on one day an increase of 0.08 pH and Subject 10, a decrease of 0.06 pH. In both of these marked changes in pH the $CO_2$ change was negligible. It is to be noted that on the eleven days there were only two decreases in pH while there were seven increases of from 0.01 to 0.03, and one unusual increase of 0.08.

*Changes in Acid-Base Balance during Digestion.*—It was formerly generally considered that during digestion the $BHCO_3$ content of the blood increases because of base freed from BCl, when the Cl as HCl is secreted into the stomach. The evidence for this concept

was based on increased $CO_2$ tension and capacity. The present study furnishes additional data concerning this problem. In nine of the experiments comparative values for before and after lunch are available. By the time of the first determination following lunch, all but two subjects showed an increase of $CO_2$ content although it was only 1 volume per cent in two instances. In these two exceptions there was a most decided drop in $CO_2$ content (3.5 and 5 volume per cent). The pH curves showed considerable variation. In five there was a slight decrease in pH; in two, a slight increase; and in three, a marked increase. Of the $CO_2$ tensions calculated for these values, four were higher and four lower than the value obtained before lunch. There was no change in two cases.

It is evident that any influence of digestion must be extremely variable because the rate of digestion is influenced by the nature of the meal such as different amounts of fat, etc. Increased $BHCO_3$ in the blood, due to base freed when chlorine as HCl is secreted into the stomach will begin to be neutralized as soon as base secretion takes place into the duodenum. It is well known that the first stimulus of duodenal secretion occurs in the early stages of gastric digestion. In later stages of digestion as increasing amounts of base go into the duodenum it is to be expected that the $BHCO_3$ content of the blood would decrease. This supposition is supported by our curves since, in those individuals who show an increase in both pH and $CO_2$ content shortly after the noon meal, there is a swing back toward the premeal condition late in the afternoon.

In general however our evidence does not support the general view that during digestion there is a constant increase in the blood of base bound as $BHCO_3$ but indicate that the changes due to digestion are so great but that they may be masked easily by changes due to other factors. Thus our data support the finding of Jansen and Karbaum. They found that the pH of the blood was not constant and concluded that the change in $BHCO_3$ during digestion was not due to secretion of HCl into the stomach, but was due to mobilization into the blood of stored base during intermediary metabolism.

The behavior following the evening meal is also variable. Of the twelve cases available for analysis only five show an increase in $CO_2$ content with seven showing a decrease. (One of these,

Subject 9, confused the results by running, see "Exercise.")
The $CO_2$ tension showed a tendency to decrease. After lunch
there were as many instances of increased as of decreased $pCO_2$
(four each). After the evening meal, one subject showed no
change, three showed increase, and eight showed a decrease in $CO_2$
tension.

*Exercise.*—Unusual activity is known to have occurred in two
instances. Subject 4 suddenly realized that he was late for his
after lunch sample and ran across the ·campus to the laboratory.
He was still breathing heavily and his pulse was rapid when the
blood was taken. His serum $CO_2$ content was 17 volume per cent
less than at 10 a.m., but his pH was only 0.02 higher. Subject 9,
on the way to the laboratory at 7 p.m. ran the last block to escape
a shower but waited about one-half hour before the sample was
taken. The serum $CO_2$ content was 9 volume per cent less with a
pH 0.02 higher than before dinner. This is an example of the
manner in which exercise may mask any digestion effects on the
acid-base condition of the blood. In both cases the exercise was
not severe and the lactic acid produced was taken care of by the
alkali reserve with but little change in pH.

*Fatigue.*—Fatigue due to extended laboratory work seems to
have little influence on the acid-base condition. The authors
(7, 8) worked under forced draft all day and evening up to the
time of taking the last sample. In both cases the evening curve
was in accord with the average of the group, and without note-
worthy variation.

*Changes in Fibrin.*—One constant phenomenon noted in connec-
tion with digestion was the marked increase in the size of the fibrin
clot. It was necessary to centrifuge much longer the blood drawn
after the meals (except breakfast) and occasionally the clot had to
be loosened with a glass rod and the blood recentrifuged. We
have no data as to whether this is due to a change in colloidal
aggregation of the fibrin or an actual increase in the amount of
fibrin and we have not been able to find satisfactory evidence re-
garding it in the literature. This point is being studied further.

*The Day as a Whole.*—The most striking result and the one that
was least expected is the definite increase in pH; *i.e.*, in the alkalin-
ity of the blood later in the day. The increase may be interrupted
by temporary fluctuations but with one exception, Experiment 3a,

the final pH at about 10 p.m. was from 0.01 to 0.07 pH more *alkaline* than in the morning. In this one case it was more alkaline at 6 p.m. and the final determination (unfortunately there was no determination between 6 and 10) was made after the subject had been dozing in a chair from some time. It has been established (Kunze, Endres) that the blood becomes more acid during sleep. The present observation of increased alkalinity during the day fits in well with this since it is obvious that the morning acid-base condition must be reestablished during the night. We have additional data to support Kunze and Endres obtained on separate experiments on two of these same subjects, Subjects 1 and 3. In both, the pH at 3 a.m. was 0.03 more acid than at retiring.

The three subjects, Subjects 1, 3, and 5, who were each studied on two separate days showed no individual regularity in their acid-base fluctuation.

### DISCUSSION.

*Digestion.*—It appears that the influence of digestion on the acid-base condition of the blood is easily neutralized or masked by other factors. In general the results from this series may be summarized thus: There is little change following breakfast, a tendency toward increased $CO_2$ content and higher pH after the noon meal, which is followed by lower pH and $CO_2$ content in the late afternoon; the evening meal may be followed by changes in either direction.

Since activity was not controlled in these experiments it is obvious that the data show the conditions resulting from all concurrent factors. It is planned to continue this study further with the activity controlled.

*Activity of Respiratory Center.*—The sharp changes recorded in these curves may be easily explained by transitory conditions but there is a general tendency toward an increased alkalinity of the blood as the day advances. For years it was a problem whether the pH or the $CO_2$ tension *of the blood* controlled respiration with gradual realization that it was either, neither, or both, depending upon the individual and upon whether the stimulus originated from the blood or in the respiratory center itself (Gesell (15)). Certainly in the normal under the conditions of the study, the acid-base condition, pH, and $CO_2$ content, must be considered as the resultant of the activity of the body as a whole. The marked

fluctuation that occurs in both pH and $CO_2$ tension would suggest that the respiratory center becomes more sensitive later in the day. However, the daily activities, deliberately uncontrolled as they were in this study, vary too much to allow deductions as to the influence of any single factor.

*Relation to So Called Normal Area.*—One practical conclusion is of importance in clinical work; *viz.*, that pH and $[CO_2]$ studies must be done under basal conditions. Some of the values obtained late in the day in these subjects fall outside the normal area (preceding paper).

It is to be noted that in the case of one individual all but one afternoon determination lie outside our normal area. This individual is of the excitable, mentally alert, constantly active type and on this day was probably constantly overventilating. His $CO_2$ tension varied between 29 and 36 mm.

### SUMMARY.

Studies have been made of the acid-base variation (pH and $CO_2$ content and tension) in blood sera on normal individuals throughout the course of an ordinary day. The pH increased from early morning, before rising, to late evening.

The increase varies from 0.01 pH to 0.07 pH.

This increase is not constant but is interrupted by fluctuations due to digestion, exercise, and other factors.

The influence of digestion is discussed. Although there is a definite tendency toward increased pH and $CO_2$ content following meals there are marked exceptions and the changes are different in degree and regularity following the evening meal as compared to the noon meal.

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## PROTOCOLS.

*Subject 1.*—H. Woman, student; age 26 years, height 5 feet, 5 inches, weight 170 pounds. (a). Mar. 24, 1929. Sunday, quiet day with little activity. (b). Mar. 26, 1929. Ordinary school day. Worked in laboratory until 10 p.m.

*Subject 2.*—S. Man, student; age 22 years, height 6 feet, weight 156 pounds. Mar. 7, 1929. First determination after breakfast. School day, walked back to laboratory before last sample.

*Subject 3.*—D. Woman, student; age 24 years, height 5 feet, 8½ inches, weight 165 pounds. (a). Feb. 28, 1929. School day. In evening slept in chair from 8 until awakened for last sample. (b). Apr. 2, 1929. School day. Afternoon reading. Evening of social activity.

*Subject 4.*—G. Man, engineering student; age 20 years, height 5 feet, 6½ inches, weight 154 pounds. Mar. 8, 1929. Athlete in training. Attended classes from 8 a.m. to 4 p.m. Ran across campus to laboratory at 4 p.m. and blood sample was taken while still breathing rapidly. Spent next hour in gym. Evening of studying.

*Subject 5.*—P. Man, engineering student; age 21 years, height 6 feet, weight 168 pounds. (a). Mar. 3, 1929. Breakfast and lunch were heavy meals. This set of determinations was made on Sunday when the subject remained at home studying most of the day, walking to and from the laboratory for drawing of each blood sample. (b). Mar. 30, 1929. Engaged in usual activities incident to class work and study.

*Subject 6.*—B. Man, student; age 27 years, height 6 feet, weight 165 pounds. Mar. 13, 1929. This subject is a nervous active individual. Arose at 7, drove to hospital for ward rounds, and ate large breakfast before first sample was taken. Active day in clinic and laboratory and evening of social activity. As it was thought that this may have been an unusually stimulating day, a blood sample was taken the next morning before rising. pH = 7.44, $[CO_2]$ = 55.5 volume per cent.

*Subject 7.*—E. Woman, staff member; age 31 years, height 5 feet, 6½ inches, weight 125 pounds. Mar. 14, 1929. First sample taken after rising but before breakfast. Worked at high tension all day until last sampling.

*Subject 8.*—C. Man, staff member; age 39 years, height 5 feet, 8½ inches, weight 200 pounds. Mar. 18, 1929. Laboratory work until last sampling. Light breakfast. Heavy lunch and dinner taken to study influence of high fat in sera. Serum highly colored, and after meals very fatty and opaque.

*Subject 9.*—W. Woman, secretary; age 22 years, height 5 feet, 5 inches, weight 125 pounds. Mar. 22, 1929. After dinner ran 2 blocks, then rested 20 minutes before blood sample. Attended theatre until last sampling.

*Subject 10.*—R. Man, staff member; age 33 years, height 5 feet, 6 inches, weight 130 pounds. Apr. 5, 1929. Usual laboratory activity. In evening read until last sample. No unusual occurrence to account for peculiar nature of results for this day. Every determination checked by both methods. This person always has a highly colored serum which is always opaque after meals.

# FURTHER FINDINGS ON INVERTASE FROM HONEY.*

By PHILIPPOS E. PAPADAKIS.

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The study of invertase from honey has brought to light a very interesting case of an invertase which differs from that obtained from yeast in several ways.

(*a*) Nelson and Cohn (1) observed that honey invertase shows a characteristic difference from yeast invertase in the beginning of the hydrolysis of sucrose. They pointed out that there is an increase in the inversion rate soon after the start of the reaction which persists until 20 per cent of the sucrose present has been hydrolyzed. When the general course of sucrose hydrolysis by honey invertase is expressed graphically, as degrees change in rotation per time, the curve shows a concave upwards bend in the beginning of the hydrolysis. This bend in the beginning of the sucrose hydrolysis curve is characteristic of honey invertase but not of invertase from yeast.

(*b*) Honey invertase is activated by β-glucose while yeast invertase is not. Nelson and Sottery (2) showed that small amounts of added glucose increase the velocity of sucrose hydrolysis by honey invertase. From their results they concluded that the increase in the inversion rate soon after the start of sucrose hydrolysis which was observed by Nelson and Cohn was due to β-glucose which resulted from the hydrolysis of sucrose. This activation by β-glucose is characteristic of honey invertase but not of invertase from yeast.

(*c*) Invertase preparations from honey do not hydrolyze raffinose (3), while invertase preparations from yeast hydrolyze raffinose. The aim of the present investigation is to obtain additional information concerning honey invertase and its properties.

Since Nelson and Sottery (2) found that β-glucose activated honey invertase, it was interesting to examine whether other aldoses such as pentoses increase the rate of sucrose hydrolysis by honey invertase. With this idea in mind the present author submits the results of a limited number of experiments in Table I. The method of procedure was similar to that used by Nelson and Sottery (2). The honey invertase preparations were made according to the procedure described below.

*Preparation of Honey Invertase.*—The preparation included preliminary treatment involving the solution of honey from buckwheat honeycombs in water and precipitation of the enzyme by alcohol according to Cohn and Nelson (1) with this modification; that before precipitation by alcohol the pH was adjusted to 5.7, which is the pH of optimum activity for honey invertase. The precipitate from the alcohol treatment was dissolved and to the solution proteolytic enzymes were added. After dialysis the honey invertase was adsorbed on alumina, eluted, and dialyzed. The preparation was kept in the ice box for 2 years, then dialyzed again against distilled water in the ice box for 4 days.

The results listed in Table I represent hydrolyses, by invertase from honey, of 10 per cent sucrose solution, containing small amounts of an added pentose. In each case control hydrolyses were run without the presence of pentose.

The results of the experiments given in Table I show that d-arabinose, l-arabinose, and mutarotated xylose at the specified concentrations and under the experimental conditions used, exert a small retardation and do not give an acceleration analogous to that of β-glucose. The results do not support the suggestion advanced by von Euler and Josephson (4), that the activation of honey invertase by glucose may be analogous to the activation by means of aldoses, of yeast invertase which has been partially inactivated by m-chloroaniline. According to the above authors, yeast invertase has an aldehyde group which reacts with the amino group of the m-chloroaniline and as a result of this reaction the yeast invertase molecules become inactive. The activity of the yeast molecules is partly regained when the inactivated molecules are treated with a sugar containing a free aldehyde group such as glucose, galactose, or maltose. Von Euler and Josephson think that there may be some material accompanying the enzyme in the

honey invertase preparations used by Nelson and Sottery, which retards the enzyme and that when glucose is added to the hydro-

TABLE I.

*Rate of Hydrolysis of 10 Per Cent Sucrose Solutions by Honey Invertase.*

Control for *d*-arabinose; pH 5.67; temperature 25°.

| | Experiment 1. | | | | Experiment 2. | | | | Experiment 3. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Time, *min* | 79 | 166 | 215 | 388 | 66 | 133 | 308 | 432 | 47 | 106 | 138 |
| Change in rotation, *degrees.* | 0.56 | 1.155 | 1 545 | 2.92 | 0.355 | 0 88 | 2 30 | 3 29 | 0.235 | 0.645 | 0.885 |

0.5 per cent *d*-arabinose; pH 5.67; temperature 25°.

| | Experiment 1. | | | | Experiment 2. | | |
|---|---|---|---|---|---|---|---|
| Time, *min* | 51 | 117 | 196 | 381 | 89 | 167 | 288 |
| Change in rotation. *degrees.* | 0 24 | (0.656) | 1.24 | 2.64 | 0.46 | 1.025 | 1.94 |

Control for xylose; pH 5.84; temperature 25°.

| | Experiment 1. | | | | Experiment 2. | | |
|---|---|---|---|---|---|---|---|
| Time, *min.* | 39.5 | 101 | 231 | 327 | 33 | 113 | 206 | 320.5 |
| Change in rotation, *degrees* | 0.62 | 2.06 | 4.99 | 6.56 | 0.51 | 2.38 | 4.51 | 6.54 |

Mutarotated xylose; pH 5.84; temperature 25°.

| | 0.8 per cent xylose. | | | | 0.393 per cent xylose. | | |
|---|---|---|---|---|---|---|---|
| Time, *min.* | 45 | 133 | 228 | 346 | 37 | 90 | 178.8 | 288.5 |
| Change in rotation. *degrees* | 0.70 | 2.685 | 4.73 | 6.69 | 0.59 | 1.73 | 3.79 | 5.77 |

Control for *l*-arabinose; pH 5.9; same as control hydrolysis for xylose.

| | | | | |
|---|---|---|---|---|
| Time, *min.* | 13 5 | 30 | 44 | 61 |
| Change in rotation, *degrees.* | 0.235 | 0.48 | 0.76 | 1 09 |

0.5 per cent mutarotated *l*-arabinose; pH 5.9.

| | | | | |
|---|---|---|---|---|
| Time, *min.* | 44.5 | 91 | 170 | 224 |
| Change in rotation, *degrees.* | 0.72 | 1.72 | 3.68 | 4.58 |

lyzing sucrose solution, its aldehyde group reacts with the accompanying retarding material, thereby leaving the enzyme free to react with sucrose.

Von Euler and Josephson, however, must have overlooked the fact observed by Nelson and Sottery that $\alpha$-glucose showed no activating influence similar to that produced by the $\beta$ modification; furthermore the results shown in Table I dealing with the in-

TABLE II.

*Influence of Mercuric Chloride on Honey Invertase. Hydrolysis of 10 Per Cent Sucrose Solutions at Different pH.*

No mercuric chloride.          0.03 units mercuric chloride.

pH = 5.72.

| Time, *min*.... | 56 | 167 | 241 | 317 | 398 | 525 | 58 | 169 | 242 | 319 | 399.5 | 536 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Change in rotation, *degrees*...... | 1.01 | 3.59 | 5.07 | 6.40 | 7.62 | 9.11 | 0 97 | 3.03 | 4.19 | 5.17 | 6 07 | 7.19 |

pH = 5.03.

| Time, *min*.... | 62 | 178 | 252 | 350 | 453 | 544 | 64 | 185 | 254 | 350 | 494 | 666 | 1265 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Change in rotation, *degrees*...... | 0.90 | 3.12 | 4 36 | 5.92 | 7.27 | 8.30 | 0.68 | 1 54 | 1.83 | 2.09 | 2.39 | 2.64 | 3.31 |

pH = 4.23.

| Time, *min*.... | 67 | 272 | 359 | 466 | 647 | 67 | 273 | 638 | 1266 | 2008 |
|---|---|---|---|---|---|---|---|---|---|---|
| Change in rotation, *degrees*...... | 0.54 | 2.55 | 3.41 | 4.46 | 5.97 | 0 21 | 0.47 | 0.80 | 1.40 | 2.15 |

pH = 6.51.

| Time, *min*.... | 49 | 116 | 173 | 233 | 44 | 111 | 169 | 220 |
|---|---|---|---|---|---|---|---|---|
| Change in rotation, *degrees*. . ... | 0.74 | 2.15 | 3.31 | 4.43 | 0.62 | 1.86 | 2.95 | 3.83 |

fluence of *d*-arabinose, *l*-arabinose, and xylose, which are also aldoses, show no accelerating influence.

If von Euler and Josephson's explanation of the effect of Nelson and Sottery's observation concerning the influence of $\beta$-glucose on honey invertase is correct, then it is surprising to find that these other aldose sugars do not also exert similar influence.

*Retarding Influence of Mercuric Chloride and α-Methylglucoside on Sucrose Hydrolysis by Honey Invertase. Effect of β-Glucose on Sucrose Hydrolysis by Honey Invertase, in Presence of the Above Retardants.*

It has been observed by various authors that mercuric chloride manifests a marked retarding influence on the hydrolysis of sucrose by yeast invertase. The following investigation was undertaken

TABLE III.

*Influence of β-Glucose on Honey Invertase. Hydrolysis of 10 Per Cent Sucrose Solutions in Presence of 0.03 Units of Mercuric Chloride.*

Control for β-glucose; pH 5.84.

|  | Experiment 1. | | | | Experiment 2. | | |
|---|---|---|---|---|---|---|---|
| Time, *min.* | 86 | 216 | 281 | 305 | 78 | 183 5 | 277 |
| Changes in rotation, *degrees.* | 1 99 | 4 88 | 6 94 | 7.77 | 1 68 | 3 89 | (5 33) |

0 5 per cent β-glucose; pH 5.84.

|  | Experiment 1. | | | | Experiment 2. | | |
|---|---|---|---|---|---|---|---|
| Time, *min.* | 33 | 109 | 237 | 327 | 54 | 145 | 243 | 298 |
| Change in rotation, *degrees* | 1 12 | 3 09 | 5 26 | 6 15 | 1.75 | 3.84 | 5 36 | 6 16 |

No β-glucose; pH 5.03.

| Time, *min.* | 26 | 71 | 96 | 170 | 247 | 533 |
|---|---|---|---|---|---|---|
| Change in rotation, *degrees* | 0 24 | 0 66 | 0 89 | 1.32 | 1 57 | 2 20 |

0.5 per cent β-glucose; pH 5.03.

| Time, *min.* | 28 | 70 | 93 | 166 | 239 | 536 |
|---|---|---|---|---|---|---|
| Change in rotation, *degrees* | 0 52 | 1 10 | 1.37 | 1.88 | 2 20 | 2.82 |

with an object to study the influence of mercuric chloride on sucrose hydrolysis by honey invertase at various pH.

Tables II to IV give the results of the experiments which show the influence of 0.03 units of mercuric chloride (1 cc. of saturated mercuric chloride per 100 cc. of solution was taken as a unit by Wallace (5)) on the rate of hydrolysis of a 10 per cent sucrose solution by honey invertase at different pH.

The results recorded in Table II indicate that: (a) At pH 5.72 the retardation caused by mercuric chloride on the rate of sucrose

hydrolysis by honey invertase is very small; and the characteristic bend of the sucrose hydrolysis curve by honey invertase is not eliminated. (b)  The retardation increases as the pH decreases to 4.23 and the shape of the curve changes.  In the case of yeast invertase Wallace (5) showed that the retarding effect of  mercuric

TABLE IV.

*Influence of β-Glucose on Honey Invertase.  Hydrolysis of 10 Per Cent Sucrose Solutions in Presence of α-Methylglucoside.*

1 per cent α-methylglucoside; pH 5.84.

| Time, *min* | 57.5 | 125 | 217 | 337 |
|---|---|---|---|---|
| Change in rotation, *degrees* | 1.12 | 2.79 | 4.86 | 6 95 |

1 per cent α-methylglucoside; 0.5 per cent β-glucose; pH 5.84.

| | Experiment 1. | | | | Experiment 2. | | |
|---|---|---|---|---|---|---|---|
| Time, *min*. | 35 5 | 63 | 127.5 | 324 | 43 | 92 | 211 |
| Change in rotation, *degrees* | 1.08 | 1.84 | 3.66 | 6 93 | 1.30 | 2.65 | 5.15 |

1 per cent α-methylglucoside; no β-glucose; pH 5.03.

| Time, *min* | 40 | 90.5 | 146 | 233 | 258 | 352 | 446 |
|---|---|---|---|---|---|---|---|
| Change in rotation, *degrees* | 0.44 | 1.09 | 1.92 | 3.20 | 3.52 | 4.79 | 5.86 |

1 per cent α-methylglucoside; 0.5 per cent β-glucose; pH 5.03.

| Time, *min* | 20 | 64 | 123 | 207 | 275 | 357 | 450 |
|---|---|---|---|---|---|---|---|
| Change in rotation, *degrees* | 0.35 | 1.12 | 2.08 | 3.29 | 4.20 | 5.15 | 6.20 |

No α-methylglucoside; pH 4.2.

| Time, *min* | 52 | 168 | 315 | 435 |
|---|---|---|---|---|
| Change in rotation, *degrees* | 0.36 | 1.35 | 2.68 | 3.76 |

1 per cent α-methylglucoside; pH 4.2.

| Time, *min* | 48 | 167 | 308 | 421 |
|---|---|---|---|---|
| Change in rotation, *degrees* | 0.26 | 0.99 | 2 00 | 2.85 |

chloride on the rate of hydrolysis became less with a  change of pH from 5.0 to 3.0.

Since mercuric chloride at pH 5.03 and at pH 4.2 changes the shape of the sucrose hydrolysis curve by honey invertase and eliminates the characteristic increase in reaction  velocity, it was

thought advisable to investigate whether or not β-glucose shows its accelerating effect on honey invertase in the presence of mercuric chloride. The experiments recorded in Table III indicate the influence of β-glucose on hydrolysis of sucrose by honey invertase in the presence of mercuric chloride.

The results, given in Table III, indicate that β-glucose accelerates the sucrose hydrolysis by honey invertase in the presence of 0.03 units of mercuric chloride both at pH 5.84 and at pH 5.03.

Sottery (6) observed that α-methylglucoside has only a slight retarding effect on the velocity of sucrose hydrolysis by honey invertase, and that the glucoside does not eliminate the characteristic initial increase in reaction velocity. The above author tried the effect of α-methylglucoside at pH 5.75 only. The fact that the retarding effect of mercuric chloride varies by varying the pH suggests that the effect of α-methylglucoside at different pH should be tried as well as the influence of β-glucose on the sucrose hydrolysis by honey invertase in the presence of α-methylglucoside. To test this the experiments which are given in Table IV were performed.

The results recorded in Table IV indicate that β-glucose accelerates the sucrose hydrolysis by honey invertase in the presence of α-methylglucoside and that at pH 5.84, 5.03, and 4.2 α-methylglucoside does not retard much and does not change the characteristic sucrose hydrolysis curve.

### SUMMARY.

The results of the present investigation seem to indicate that in the case of honey invertase:

1. Sucrose hydrolysis is not activated by the pentoses, mutarotated xylose, d-arabinose, and l-arabinose.

2. Mercuric chloride does not retard the rate of sucrose hydrolysis much at pH 5.7, but the retardation is more pronounced as the pH decreases from 5.7 to 4.23.

3. The influence of β-glucose is independent of the presence of mercuric chloride.

4. β-Glucose accelerates the sucrose hydrolysis by honey invertase in the presence of α-methylglucoside. α-methylglucoside does not retard much and does not change the shape of the curve when the pH is varied from 5.84 to 4.2.

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# THE CHEMICAL STUDY OF BACTERIA.

## XXIX. A PROXIMATE ANALYSIS OF A DEFATTED RESIDUE OF AVIAN TUBERCLE BACILLI.

BY ALICE G. RENFREW.[*]

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### INTRODUCTION.

For some time a chemical study of tubercle bacilli (Strain H-37) has been in progress in this laboratory. A recent extensive study of the lipoids has been made by Anderson (1). Aside from that investigation, attention has been centered chiefly on the nitrogen constituents of the cell—more particularly proteins and nucleic acid. The nitrogen content of the defatted cell is 10 to 11 per cent, indicating a large proportion of protein, for which the distribution of amino acids has been determined (2, 3). The aqueous or saline extracts of the cell residue yield only 1 to 2.5 per cent of protein, but in addition, contain nitrogen in dialyzable, non-protein combination (4). This water-soluble protein has the characteristic biological activity associated with tuberculin preparations (4, 5). A second and larger yield of protein may be obtained by extraction with 0.5 per cent sodium hydroxide solution. Van Slyke analyses indicate a higher content of basic amino acids for this protein than for the water-soluble protein (6). However, since the protein from the alkaline extract has very little tuberculin potency in skin tests, it has received relatively little attention.

The findings with regard to the constitution of tuberculinic acid place it among the animal nucleic acids. The purines adenine and guanine were identified by Levene (7) and by Long (8). Johnson and Brown isolated the pyrimidines thymine and cytosine (9), and later Johnson and Coghill identified methylcytosine (10). As further evidence for the classification of tuberculinic acid, the

presence of a hexose sugar was demonstrated by the isolation of levulinic acid (11).

In a recent paper Johnson and Renfrew (12) have reported a carbohydrate obtained from the aqueous extract of the defatted Strain H-37 residue. In the present paper we note, in relation to the analysis of the avian residue, the isolation of a carbohydrate fraction from the alkaline extract of the bacillus, Strain H-37; this preparation has a higher pentose content (as determined by a furfural distillation) than the carbohydrate from the aqueous extract. Laidlaw and Dudley (13) have previously described a carbohydrate which gave specific precipitin reactions; their product was obtained from a human strain of tubercle bacilli.

During the course of the above investigations, a general scheme of procedure for the analysis of bacteria was developed by Johnson (14) and later elaborated by Johnson and Anderson (15). This procedure has been followed in a large measure in the study of the avian strain of tubercle bacilli.

<center>EXPERIMENTAL.</center>

The avian tubercle bacilli (Strain 531) were grown on Long's synthetic medium (16) by the H. K. Mulford Company at Glenolden, Pa. A chemical study of weekly samples from this growth (Lot II) has already been reported (17). More recently Anderson (18) has described the extraction of lipoid from these organisms by digestion in a 50 per cent alcohol-ether mixture and in chloroform. The defatted residue (Fraction G of the chart outlined by Johnson (14)), was finally dried by passing an air current over thin layers of the cellular material.

200 gm. units of the air-dried residue were used in the following study. Since this material lost 17 per cent in weight when dried over dehydrite at 60° *in vacuo*, percentage yields are calculated on the basis of 166 gm., dry weight.

*Preparation of Avian Water-Soluble Protein.*—166 gm. of defatted, desiccated, and finely pulverized bacilli were allowed to macerate for 6 hours in a ball mill with 600 cc. of water and a little toluene. About 380 cc. of a densely milky liquid were recovered during centrifugalization of the macerated paste, and the moist residue was returned to the ball mill for a second extraction. The combined extracts were clarified by filtration through care-

fully washed paper pulp and through a Berkefeld candle. A third extract was clarified and analyzed separately to determine the completeness with which water-soluble substances had been removed.

The Berkefeld filtrate (Fraction R (14)) had a pH of 6.5, corresponding closely to the reaction of similar extracts of the human organism. The solution contained 0.70 gm. of nitrogen or 4.5 per cent of the total nitrogen of the defatted cells. Maximum flocculation of protein was obtained in the presence of 2 to 4 per cent acetic acid. The water-soluble protein, thus prepared, did not reduce Benedict's solution either before or after hydrolysis. The yield was about 0.48 gm. (0.38, 0.58, 0.56, 0.42 gm.) with a nitrogen content of 14.46 per cent.

*Isolation of Carbohydrate Fraction from Protein Filtrate.*—In the study of the tubercle bacillus (Strain H-37) a carbohydrate (12) was separated from the acetic acid filtrate after the removal of protein. A similar, but much smaller, amount of material, precipitable with basic lead acetate, has been found in the corresponding aqueous extract of the avian organism.

The acetic acid solution (Fraction T) was first concentrated to a volume of 150 to 200 cc. *in vacuo* at 40°. A precipitate, obtained at this point after the addition of 2 volumes of 95 per cent alcohol, was removed and is recorded in this paper as avian Fraction Aq-3. The alcoholic supernatant liquid was concentrated to 75 cc. and treated with a 20 per cent lead acetate solution till no further precipitation occurred. The final concentrate at a volume of 30 cc. was precipitated with basic lead acetate (Goulard's solution (19)) and excess ammonium hydroxide. 1.9 to 2.3 gm. or a 1.4 per cent yield of carbohydrate was recovered from the basic lead acetate precipitate after removal of the lead as sulfide and the dehydration of the concentrated syrup with absolute alcohol.

The slight reducing properties of the avian carbohydrate were greatly increased after acid hydrolysis. 0.0362 gm. was heated with 5 per cent sulfuric acid for 8 hours on a boiling water bath. The reducing value of this hydrolysate as determined by the Shaffer-Hartmann micro method was equivalent to 0.0177 gm. of glucose—or 48.8 per cent of the weight of carbohydrate taken. A sample of the carbohydrate from Strain H-37 bacilli was hydrolyzed at the same time and gave 52 per cent of reducing sugars

calculated as glucose.   That hydrolysis is rather difficult is shown by the values for solutions treated 5 hours with 0.5 N hydrochloric acid at 80–100.°   The glucose equivalent for the avian sugar was 37 per cent and for the human 39 per cent.

The avian carbohydrate contains small amounts of both nitrogen and phosphorus and gives 8.2 per cent pentose as calculated from the furfural phloroglucinol obtained in a furfural distillation.

*Avian Fraction Aq-3.*—The avian Fraction Aq-3 was a white powder, only partially soluble in water.   A clear solution, which was easily obtained by the addition of a little 0.1 N hydrochloric acid, clouded with a rather gelatinous precipitate when the reaction was made neutral or alkaline.   A solution of Fraction Aq-3 gave a fairly heavy precipitate with molybdate reagent but did not reduce Benedict's solution.   Tests for glycogen and protein were negative.   The hydrolyzed solution of Fraction Aq-3 was an actively reducing solution and gave a heavy precipitate when tested with molybdate reagent.   A Weidel test was negative.

*Preparation of Avian Alkali-Soluble Protein.*—The insoluble residue from the aqueous extraction was not dried, but was again returned to the ball mill with 500 cc. of dilute alkali.   After two extractions with 0.5 per cent sodium hydroxide, the pasty residue was macerated with 400 cc. of water.   The two alkaline solutions were combined, clarified, and filtered through a Berkefeld candle.   The nitrogen content of the Berkefeld filtrate was 1.26 gm. of nitrogen, or 8.1 per cent of the total nitrogen in the original defatted bacilli.   This solution had a pH of about 9.2.   Heavy flocculation of protein followed the addition of acetic acid to a concentration of 0.3 to 0.5 per cent.

The alkali-soluble protein weighed about 2.9 gm. and on analysis gave 15.86 per cent of nitrogen.   This preparation gives a Molisch reaction but does not reduce Benedict's solution either before or after hydrolysis.   The final 400 cc. of water washings of the residue contained 0.8 gm. of protein.

Table I indicates comparative yields of protein and carbohydrate from avian and from human bacilli, which have been defatted in accordance with the method described by Anderson (18).

The supernatant solutions after acetic acid precipitation of the alkali-soluble protein were concentrated to small volume and fractionally precipitated with alcohol.   After acid hydrolysis

these fractions, designated Fractions $O_3$ and $O_4$ have marked reducing properties.

*Bacterial Residue Insoluble in 0.5 Per Cent NaOH (Residue P).*—The weight of the bacterial residue recovered from the alkaline extraction was about 100 to 110 gm. This residue contains 10.05 per cent of nitrogen, which may be regarded as largely protein nitrogen in view of the satisfactory results for amino acid determinations (2) and Van Slyke (3) analyses when the insoluble bacterial residue of Strain H-37 is treated as whole protein. The avian residue gives positive biuret and Millon tests and after hydrolysis reduces Benedict's solution. An attempt was made to isolate an osazone from the hydrolysate of the avian residue, Residue P, after the removal of amino acids with mercuric acetate. Only a very small amount of a yellow osazone with the apparent

TABLE I.

*Comparative Yields of Bacterial Products.*

| 100 gm unit. | H₂O-soluble protein | Carbohydrate from aqueous solution | Alkali-soluble protein. |
|---|---|---|---|
| | *gm* | *gm.* | *gm.* |
| Avian (Strain 531) . . . . . . . . . . . . | 0.35 | 1.4 | 2.9 |
| Human (Strain H-37) . . . . . . . | 0.50 | 3.9 | 9.6 |

properties of phenylglucosazone was obtained. This osazone, which was soluble in alcohol but insoluble in acetone, melted at 175–180°; a mixture of phenylglucosazone with the unknown product melted at 189–192°.

*Liberation of Additional Quantities of Lipoid after Treatment of Defatted Residue G or Residue P.*—It has been previously noted by Long (20) and others that even after exhaustive extraction with fat solvents, the bacterial residue still contains lipoid materials which may be brought into soluble form by acid hydrolysis. 10 gm. portions of the defatted avian Residue G (14) were hydrolyzed for 2 hours with 20 per cent hydrochloric acid. The residue (about 2 gm.) from the filtered solution was thoroughly washed, dried, and extracted with chloroform. Evaporation of the chloroform leaves a waxy substance which is soluble in chloroform and in ether but insoluble in acetone or alcohol. For the avian bacillus

this lipoid fraction represented 15 per cent of the weight of the residue hydrolyzed and for the human about 8 per cent. A Liebermann-Burchard test gave no evidence of cholesterol in the avian wax.

Only 11 per cent of lipoid was obtained after hydrolysis of Residue P (14), which had been freed from substances soluble in 0.5 per cent sodium hydroxide. This lower percentage yield as compared with the original bacterial Residue G would suggest a loss, as yet unaccounted for, in the chloroform-soluble fraction during alkaline extraction. There is still very little lipoid in directly soluble form in Residue P, since several successive extractions removed only 2.5 per cent of a chloroform-soluble, semitransparent, waxy material.

A lipoid determination was also carried out under conditions more closely resembling those described by Long (20). 10 gm. of the defatted avian Residue G were heated 2 hours on a steam bath with 70 cc. of N hydrochloric acid. The yield of 9.4 per cent of lipoid was probably low because of losses in the repeated filtration needed to clarify the chloroform solution. The wax thus obtained was of a more brittle consistency than that of the corresponding product from treatment with 20 per cent acid. Even after the chloroform solution of the lipoid had been filtered through a Berkefeld candle, the waxy product from treatment with N hydrochloric acid gave slightly positive tests for both nitrogen and phosphorus. A Liebermann-Burchard test for cholesterol was negative, and the chloroform layer in the Salkowski test reddened only after 6 to 8 hours.

### DISCUSSION.

Certain differences in the proportionate amounts of the fractions of the avian bacillus as compared with the human strain are brought out in the above chemical study. Under the same experimental conditions distinctly less carbohydrate, precipitable with basic lead acetate, was obtained from the avian aqueous extract: 1.4 per cent from the avian organism and 3.9 per cent from Strain H-37, or a 1:3 ratio. Since this product is rather easily extracted and isolated, the difference in yields seems marked and unquestionable; however, this difference is less striking than

TABLE II.

*Nitrogen Distribution in Defatted Avian Tubercle Bacilli.*

| | Avian Strain 531. | | | | | | Human Strain H-37. | | |
| | Experiment 1 | | | Experiment 2 | | | | | |
| | gm. | per cent N | per cent of total N | gm. | per cent N | per cent of total N | gm. | per cent N | per cent of total N |
|---|---|---|---|---|---|---|---|---|---|
| Defatted bacteria | 100 | 9.33 | 100 | 100 | 9.33 | 100 | 100 | 10.72 | 100 |
| Aqueous extract. | | | | | | | | | |
| Protein | 0.288 | 14.66 | 0.44 | 0.293 | | 0.45 | 0.5 | 13.78 | 0.64 |
| Non-protein N* | | | 4.44 | | | 4.1 | | | 10.6 |
| Total N content of extract | 0.45 | | 4.8 | 0.40 | | 4.6 | 1.21 | | 11.3 |
| Alkaline extract. | | | | | | | | | |
| Protein | 2.2 | 15.86 | 3.6 | 1.86 | | 3.1 | 9.6 | 16.24 | 14.9 |
| Non-protein N* | | | 4.5 | | | 5.3 | | | 4.1 |
| Total N content of extract | 0.76 | | 8.1 | 0.79 | | 8.4 | 2.04 | | 19.0 |
| Insoluble residue† | 74 | 10.05 | 79.7 | 68 | | 73.2 | 35 | 12.38 | 40.4 |
| Total N accounted for | | | 92.6 | | | 86.2 | | | 70.7 |

\* Non-protein nitrogen calculated by difference.

† Residue nitrogen was not accurately determined in consequence of unavoidable losses in recovery of residue.

the comparative 1:6 values determined for copper-reducing substances in the study of the culture medium of these two organisms.

The differences in nitrogen distribution for the avian and human strains may be seen in Table II. The data for the human strain are average results from extractions of unit lots of Strain H-37 bacilli defatted by Anderson (1). A consideration of Table II shows a definitely lower percentage of water-soluble non-protein nitrogen for the avian bacillus, and a smaller quantity of alkali-soluble protein as compared with Strain H-37. It is interesting to note that the amount of alkali-soluble protein extracted from the avian bacillus closely parallels the results obtained by Coghill and Bird (21) for a non-pathogenic organism, the timothy bacillus. The amounts of water-soluble protein, extracted from exhaustively defatted residues of both avian and human strains, are about the same; such very low yields, however, make quantitative comparisons unsatisfactory. A more significant comparison might be obtained if the yield of water-soluble protein were known for an avian residue treated only with ether, inasmuch as protein yields seem to be higher under these conditions (12).

It seems very possible that the lipoid which is liberated in available form only after acid treatment of the bacilli may have biological properties of marked interest. Long (20) has studied this fraction in relation to the acid-fast properties of the bacterial cell. However, we are not familiar with any investigation of this material under conditions comparable to the studies of Sabin and Doan with the phosphatides from tubercle bacilli.

The proximate chemical analysis of the aqueous and alkaline extracts of avian and human bacilli does not provide any single outstanding factor which, *ipso facto*, would serve to distinguish between the two types. There are marked similarities in the general fractions separated. Nevertheless, there are differences in the relative amounts of various fractions from the two types and a more precise chemical study may show differences in the chemical components of corresponding fractions. Whether the substances isolated will have biological specificity remains to be determined and the character of further chemical work must in a large measure depend upon the biological findings.

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SUMMARY.

1. The defatted avian residue has been subjected to extraction with water, sodium chloride solution, and a solution of 0.5 per cent sodium hydroxide. The nitrogen distribution under these conditions has been determined.

2. The protein comparable to Protein 304 (12) of the human strain, the carbohydrate material from Fraction T (14) comparable to Carbohydrate 81 (12) of the human strain, the protein extracted by 0.5 per cent sodium hydroxide, and certain lipoid fractions have been isolated from the avian organism for testing.

3. The present yields of the carbohydrate fraction and the yields of the alkali-soluble protein are low in comparison with similar fractions from Strain H-37.

4. Further quantities of lipoid of a waxy character have been obtained from human and avian strains after acid treatment of bacterial residues which had previously been thoroughly extracted with fat solvents.

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# THE CONFIGURATIONAL RELATIONSHIPS OF ETHYL-
# BUTYL AND PROPYLBUTYL CARBINOLS TO
# LACTIC ACID.

## WITH A NOTE ON THE EFFECT OF UNSATURATION ON
## OPTICAL ACTIVITY.

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New York.)*

In a previous article[1] on the relationship of chemical structure
to optical activity, the conclusion was reached that the direction
of rotation of a simple secondary aliphatic carbinol can be pre-
dicted from the knowledge of its structure. Taking dextro-
methylethyl carbinol

$$CH_3$$
$$|$$
$$HCOH$$
$$|$$
$$C_2H_5$$
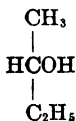
as reference substance, it was stated that all secondary carbinols
having the lighter group on the top of the projection in place of
the methyl and the heavier group at the bottom in place of the
ethyl, rotate to the right and *vice versa*. The rule was deduced
from the observation on the rotations of three carbinols of the
methylethyl series and of one of the carbinols of the ethylpropyl
series of which the configurations were established by direct chem-
ical methods. The observations have now been extended to one

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[1] Levene, P. A , and Haller, H. L , *J Biol. Chem* , **79**, 475 (1928).

additional member of the ethylmethyl series; namely, to ethyl-
butyl carbinol of the following configuration

$$
\begin{array}{c}
C_2H_5 \\
| \\
HCOH \\
| \\
C_4H_9
\end{array}
$$

and to propylbutyl carbinol

$$
\begin{array}{c}
C_3H_7 \\
| \\
HCOH \\
| \\
C_4H_9
\end{array}
$$
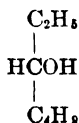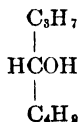
According to the rule announced by us these two carbinols should
be dextrorotatory and indeed they rotate to the right.

The set of reactions which led to the elucidation of the con-
figuration of dextro-propylbutyl carbinol was the following:

$$
\begin{array}{ccccc}
CH_3 & CH_3 & & & \\
| & | & & & \\
CH_2 & CH & & & \\
| & \| & & & \\
CH_2 & CH & CHO & CH_2OH & COOH \\
| & | & | & | & | \\
HCOH \leftarrow & HCOH \rightarrow & HCOH \rightarrow & HCOH \overset{*}{\leftarrow} & HCOH \\
| & | & | & | & | \\
C_4H_9 & C_4H_9 & C_4H_9 & C_4H_9 & C_4H_9 \\
\text{Dextro.} & \text{Levo} & \text{Dextro} & \text{Dextro.} & \text{Levo.} \\
\text{I.} & \text{II.} & \text{III.} & \text{IV.} & \text{V.}
\end{array}
$$
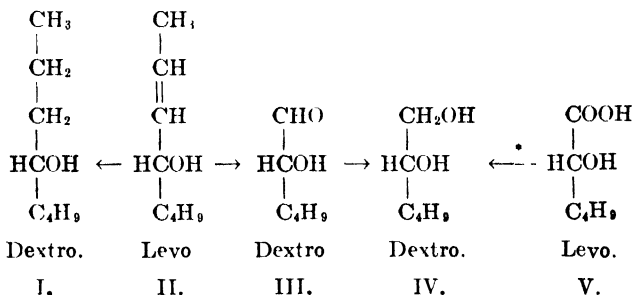
* See foot-note 1.

This series of reactions differs from similar ones previously
reported in that an additional step, (III) to (IV), has been intro-
duced.   This was made necessary by the fact that the oxidation
of the α-hydroxyaldehyde was accompanied by a high degree of
racemization, regardless of the conditions of oxidation.   On the
other hand, the reduction of the aldehyde to the glycol proceeded
smoothly without any sign of racemization.   An advantage was
gained by this additional step as it furnished information which
facilitated the elucidation of the configuration of the dextro-

ethylbutyl carbinol. This task was accomplished through the
following steps.

$$
\begin{array}{cccc}
\text{CH}_3 & \text{CH}_2 \cdot & & \\
| & \| & & \\
\text{CH}_2 & \text{CH} & \text{CHO} & \text{COOH} \\
| & | & | & | \\
\text{HCOH} \leftarrow & \text{HCOH} \rightarrow & \text{HCOH} \xrightarrow[\text{to}]{\text{Related}} & \text{HCOH} \\
| & | & | & | \\
\text{C}_4\text{H}_9 & \text{C}_4\text{H}_9 & \text{C}_4\text{H}_9 & \text{C}_4\text{H}_9 \\
\text{Dextro.} & \text{Levo.} & \text{Dextro.} & \text{Levo.}
\end{array}
$$
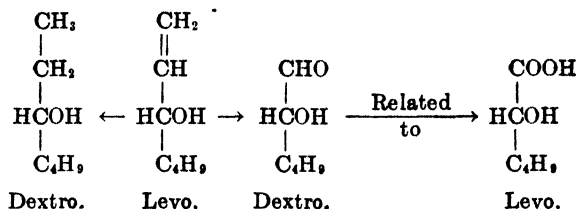
The relation between the unsaturated and saturated carbinols
observed by us is in the opposite sense from that observed by
Kenyon and Snellgrove.[2] Mindful of the usual thoroughness of
the work of Pickard and Kenyon and their associates, we have
repeated the reduction several times varying the catalyst from
colloidal palladium to platinum oxide, as well as the solvent,
employing ether and 50 per cent acetic acid. The result, how-
ever, remained the same. Besides, if the observation of Pickard
and Snellgrove is correct, then levo-ethylpropyl carbinol should be
configurationally related to levo-2-hydroxycaproic acid. This
conclusion would be contrary to the rule deduced by us from
observations reported earlier. Nevertheless, we thought it
desirable to redetermine the configuration of ethylpropyl carbinol
starting with vinylpropyl carbinol and compare the result with
the one previously obtained by another set of reactions.[3] The
present findings are given by the following set of reactions.

$$
\begin{array}{ccccc}
\text{CH}_3 & \text{CH}_2 & & & \\
| & \| & & & \\
\text{CH}_2 & \text{CH} & \text{CHO} & \text{CH}_2\text{OH} & \text{COOH} \\
| & | & | & | & | \\
\text{HCOH} \leftarrow & \text{HCOH} \rightarrow & \text{HCOH} \rightarrow & \text{HCOH} \xleftarrow{*} & \text{HCOH} \\
| & | & | & | & | \\
\text{C}_3\text{H}_7 & \text{C}_3\text{H}_7 & \text{C}_3\text{H}_7 & \text{C}_3\text{H}_7 & \text{C}_3\text{H}_7 \\
\text{Dextro.} & \text{Levo.} & \text{Dextro.} & \text{Dextro.} & \text{Levo.}
\end{array}
$$
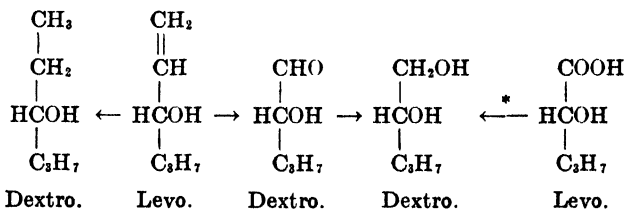
* Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **77**, 555 (1928).

Thus, in this case also, the reduction of the vinylpropyl carbinol
leads to an inversion of the direction of rotation, as in our obser-

[2] Kenyon, J., and Snellgrove, D. R., *J. Chem. Soc.*, **127**, 1169 (1925).

[3] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928).

vation on the reduction of vinylbutyl carbinol, and the conclusion regarding the configuration of the ethylpropyl carbinol deduced from this observation is in harmony with our earlier conclusion based on another set of reactions. We are inclined to believe that there was an error in the records of Kenyon and Snellgrove concerning the rotation of the ethylbutyl carbinol obtained by them from the levo-vinylbutyl carbinol.

### Influence of Double Bond.

The material on the influence of the double bond has been increased by the observations made in the course of the present investigation and the tabulation of all the results obtained by us to date permits more comprehensive conclusions than those previously possible. *A priori*, the following influences of the introduction of a double bond are possible. If a change of rotation occurs it may either bring about an exaltation in the original direction of rotation, or the change may be in the opposite direction. In the latter case, three results are possible. First, the rotation remains of the same direction and hence the value of the rotation is lower than in the parent substance; second, the direction of rotation has the opposite sign and the value is lower than that of the original; third, the direction of rotation has the opposite sign and the value is higher than that of the original. All four possibilities have been observed as is seen from the values under the following figures.

$$
\begin{array}{cc}
\mathrm{CH_3} & \mathrm{CH_2} \\
| & \| \\
\mathrm{CH_2} & \mathrm{CH} \\
| & | \\
\mathrm{CH_2} & \mathrm{CH_2} \\
| \; \leftarrow & | \\
\mathrm{CH_2} & \mathrm{CH_2} \\
| & | \\
\mathrm{HOCH} & \mathrm{HOCH} \\
| & | \\
\mathrm{CH_3} & \mathrm{CH_3}
\end{array}
\qquad
\begin{array}{cc}
\mathrm{CH_3} & \mathrm{CH_2} \\
| & \| \\
\mathrm{CH_2} & \mathrm{CH} \\
| \; \leftarrow & | \\
\mathrm{CH_2} & \mathrm{CH_2} \\
| & | \\
\mathrm{HOCH} & \mathrm{HOCH} \\
| & | \\
\mathrm{CH_3} & \mathrm{CH_3}
\end{array}
\qquad
\begin{array}{cc}
\mathrm{CH_3} & \mathrm{CH_2} \\
| & \| \\
\mathrm{CH_2} & \mathrm{CH} \\
| \; \leftarrow & | \\
\mathrm{CH_2} & \mathrm{CH_2} \\
| & | \\
\mathrm{HOCH} & \mathrm{HOCH} \\
| & | \\
\mathrm{CH_2} & \mathrm{CH_2} \\
| & | \\
\mathrm{CH_3} & \mathrm{CH_3}
\end{array}
\qquad
\begin{array}{cc}
\mathrm{CH_3} & \mathrm{CH_3} \\
| & | \\
\mathrm{CH_2} & \mathrm{CH} \\
| & \| \\
\mathrm{CH_2} & \mathrm{CH} \\
| \; \leftarrow & | \\
\mathrm{HOCH} & \mathrm{HOCH} \\
| & | \\
\mathrm{CH_3} & \mathrm{CH_3}
\end{array}
$$

| + 8.25 | + 12.25 | + 6.5 | + 2.6 | + 1.35 | + 0.15 | + 7.95 | Slightly lev |
|---|---|---|---|---|---|---|---|

| Exaltation. Sign unchanged. | | Drop in value. Sign unchanged. | | | | Drop in value. Sign changed. | |
| I. | | II. | | | | III. | |

```
CH₃      CH₃      CH₃      CH₃      CH₃      CH₂      CH₃      CH₃
 |        |        |        |        |        ‖        |        |
CH₂      CH₂      CH₂      CH₂      CH₂      CH       CH₂      CH₂
 |        |        |        |        |        |        |        |
CH₂      CH₂      CH₂      CH₂      CH₂      CH₂      CH₂      CH₂
 |   ←    |   ←    |   ←    |   ←    |   ←    |   ←    |   ←    |
HOCH     HOCH     CH₂      CH₂      HOCH     HOCH     CH₂      CH₂
 |        |        |        |        |        |        |        |
CH₂      CH       HOCH     HOCH     CH₂      CH       HOCH     HOCH
 |        ‖        |        |        |        ‖        |        |
CH₃      CH₂      CH₃      CH       CH₃      CH₂      CH₂      CH
                   |        ‖                          |        ‖
                  CH₃      CH₂                        CH₂      CH
                                                       |        |
                                                      CH₃      CH₃

+2.75   − 19 40   + 5 50  − 18 60   + 1.20   − 8.4    + 0.29   − 3.0
```

Exaltation
Sign changed

IV.

From these values it is seen (1) that an exaltation without change of sign occurred when the double bond was between carbon atom 3 and 4 from the carbinolic group; (2) that a drop in value without a change of sign occurred in those carbinols where the double bond was introduced between carbon atoms 2 and 3; (3) that a drop in value with a change of sign was observed when the double bond was between carbon atom 1 and 2 of the heavier group; and (4) that a higher value with a change of sign occurred when the double bond was between carbon atom 1 and 2 of the lighter group.

*Thus, it is evident that the influence of the double bond on the optical rotation of the normal secondary carbinols depends on two factors: First, its position in the lighter or heavier groups and second, the distance of the double bond from the asymmetric carbon atom.*

### EXPERIMENTAL.

*Relation of Propylbutyl Carbinol to 2-Hydroxycaproic Acid.*

*Octen-(2)-ol-(4).*—This carbinol[4] was obtained on condensation of crotonic aldehyde with butyl magnesium bromide in the usual manner. It distilled at 78° ±1.5°, p = 20 mm.

[4] We are indebted to Mr R E Marker for the preparation of this carbinol.

*Resolution of Octen-(2)-ol-(4).*—The acid phthalate was prepared by heating a solution of 100 gm. of the carbinol, 116 gm. of phthalic anhydride and 200 cc. of dry pyridine for 1 hour on the steam bath. The isolation and the purification of the acid phthalate was effected in the usual way.[1] The inactive acid phthalate melted at 104°.

Considerable difficulty was encountered in obtaining a crystalline alkaloid salt suitable for resolution. Finally the cinchonidine salt was obtained crystalline and this was repeatedly recrystallized from acetone. After many recrystallizations the less soluble salt was decomposed with dilute hydrochloric acid and the phthalate was isolated in the usual manner. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{24} = \frac{-1.35^{\circ} \times 100}{1 \times 17.6} = -7.7^{\circ}.$$

The phthalate was dissolved in a solution of sodium hydroxide (3 mols) and the solution was steam-distilled. The carbinol was extracted with ether and the ether extract was dried over anhydrous potassium carbonate. After removal of the ether the carbinol was distilled under reduced pressure. It boiled at 79°, p = 26 mm. It analyzed as follows:

0.1012 gm. substance: 0.2788 gm. $CO_2$ and 0.1142 gm. $H_2O$.
$C_8H_{16}O$. Calculated. C 74.95, H 12.57.
Found.       " 75.12, " 12.62.

In a 1 dm. tube without solvent $\alpha_D^{24} = -2.90^{\circ}$.
In ether the carbinol was dextrorotatory.

$$[\alpha]_D^{24} = \frac{+0.42^{\circ} \times 100}{1 \times 20} = +2.1^{\circ}.$$

Another lot of carbinol (Lot 1294), obtained as described above, had the following rotation. $\alpha_D^{25} = -3.0^{\circ}$, $l = 1$ dm.
In ether the rotation was

$$[\alpha]_D^{24} = \frac{+0.50^{\circ} \times 100}{1 \times 19.4} = +2.6^{\circ}.$$

An aliquot part of this ether solution was diluted with an equal volume of ether.  The rotation of this solution was

$$[\alpha]_D^{25} = \frac{+\ 1.00° \times 100}{2 \times 9.7} = +\ 5.2°.$$

In absolute alcohol the rotation was

$$[\alpha]_D^{25} = \frac{-\ 0.63° \times 100}{1 \times 19.0} = -\ 3.3°.$$

An aliquot part of this solution diluted with an equal volume of absolute alcohol gave the following rotation.

$$[\alpha]_D^{25} = \frac{-\ 0\ 50° \times 100}{2 \times 9.5} = -\ 2.6°.$$

*Dextro-Octanol-(4)* (*Propylbutyl Carbinol*).—7 gm. of levo-octen-(2)-ol-(4) (Lot 1294) were reduced in ether solution with hydrogen in the presence of colloidal palladium as catalyst.  After filtering off the catalyst, the ether solution was dried over anhydrous potassium carbonate.  The ether was removed and the carbinol was distilled under reduced pressure.  It boiled at 79°, p = 20 mm.  It analyzed as follows:

2 570 mg. substance: 6 945 mg. $CO_2$ and 3.180 mg. $H_2O$.
$C_8H_{18}O$.  Calculated.  C 73.85, H 13 85.
Found.       " 73.69, " 13.84.

In a 2 dm. tube $\alpha_D^{27} = +0.573° \pm 0.003$.
The rotation in absolute alcohol was

$$[\alpha]_D^{27} = \frac{+\ 0.084° \times 100}{4 \times 7.86} = +\ 0\ 27°.$$

In ether the carbinol was levorotatory.

$$[\alpha]_D^{27} = \frac{-\ 0.052° \times 100}{4 \times 9.31} = -\ 0.14°.$$

*Dextro-Hexanal-(1)-ol-(2)*  (*2-Hydroxycaproic Aldehyde*).—The octen-(2)-ol-(4) was ozonized in glacial acetic acid.  Into 3 gm. of levo-octen-(2)-ol-(4) (Lot 1294) dissolved in 10 cc. of glacial

acetic acid a stream of ozonized oxygen was passed until the
solution no longer decolorized a solution of bromine in glacial
acetic acid.  The remaining liquid was diluted with dry ether
and the ozonide was decomposed[5] in the presence of zinc.  After
filtering, the ether solution was washed with ice-cold 10 per cent
potassium carbonate solution and then with water.  The ethereal
solution was dried over sodium sulfate, the ether was removed,
and the hydroxyaldehyde was distilled.  It boiled at 60–64°, p =
1 mm.  It readily crystallized.

It analyzed as follows:

3.550 mg. substance: 7.940 mg. $CO_2$ and 3.315 mg. $H_2O$.
$C_6H_{12}O_2$.  Calculated.  C 62.07, H 10.34.
                Found.       " 60.99, " 10.44.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{24} = \frac{+\ 3.50° \times 100}{2 \times 8.8} = +\ 19.9°.$$

In subsequent experiments the aldehyde was not distilled since
it was found that after removal of most of the ether the aldehyde
crystallized readily when placed in the refrigerator.

*Dextro-Hexandiol-(1 . 2) (1 , 2-Dihydroxyhexane)*.[1]—The aldehyde
obtained as described above was reduced with sodium amalgam in
80 per cent alcohol solution.  12 gm. of levo-octen-(2)-ol-(4) (Lot
1294) were ozonized and the ozonide was decomposed as described
above.  After removal of most of the ether, the syrup was dis-
solved in 180 cc. of 80 per cent alcohol.  The solution was cooled
in an ice water bath, stirred with a mechanical stirrer, and treated
with sodium amalgam until the solution no longer reduced
Fehling's solution.  The reaction mixture was kept slightly
alkaline to litmus by the addition of 10 per cent sulfuric acid.
When reduction was complete, the reaction mixture was treated
with carbon dioxide, filtered, and the solution concentrated on the
water pump.  Absolute alcohol was added to the remaining
syrup, the solution filtered and concentrated on the water pump.

⁵ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 177 (1929).

The glycol was obtained by distillation under reduced pressure. It distilled at 85–90°, p = 1.5 mm. It analyzed as follows:

4.765 mg. substance: 10.740 mg. $CO_2$ and 4.975 mg. $H_2O$.
$C_6H_{14}O_2$. Calculated. C 61.02, H 11.95.
Found. " 61.42, " 11.68.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{+\ 1.25° \times 100}{2 \times 10.9} = +\ 5.7°.$$

The glycol was further identified by converting it into its dinaphthylurethane. This melted at 165–168°. It analyzed as follows:

0 0698 gm substance: 2 99 cc 0 1 N HCl.
$C_{28}H_{28}O_4N_2$. Calculated. N 6.14. Found. N 5 99.

In glacial acetic acid it had the following rotation.

$$[\alpha]_D^{25} = \frac{-\ 0.20° \times 100}{2 \times 1.4} = -\ 7.1°.$$

*Relation of Ethylbutyl Carbinol to 2-Hydroxycaproic Acid.*

*Hepten-(1)-ol-(3) (Vinylbutyl Carbinol).*—This carbinol[4] was obtained on condensation of acrolein with butyl magnesium bromide in the usual manner.

*Resolution of Hepten-(1)-ol-(3).*[2]—The acid phthalate of the carbinol was prepared by the usual procedure. The melting point was 66–67°.[6] The strychnine salt was prepared in 95 per cent alcohol and then recrystallized from 90 per cent alcohol. On decomposition a carbinol was obtained which distilled at 65°, p = 20 mm.

It analyzed as follows:

4.430 mg. substance: 11.855 mg. $CO_2$ and 5.130 mg. $H_2O$.
$C_7H_{14}O$ (Lot 1382). Calculated. C 73.68, H 12.28.
Found. " 72.99, " 12.95.

In a 1 dm. tube without solvent $\alpha_D^{25} = -18.5°$; $[\alpha]_D^{25} = -22.2°$. In absolute alcohol the rotation was

$$[\alpha]_D^{25} = \frac{-\ 2.73° \times 100}{1 \times 13.0} = -\ 21.0°.$$

---

[6] Kenyon and Snellgrove give 56–57°, probably a typographical error.

*In ether the carbinol had the following rotation.*

$$[\alpha]_D^{25} = \frac{-1.43° \times 100}{1 \times 12.2} = -11.7°.$$

*Dextro-Heptanol-(3)* *(Ethylbutyl Carbinol).*—5 gm. of levo-hepten-(1)-ol-(3) ($\alpha_D^{25} = -18.5°$ in a 1 dm. tube without solvent $n_D^{25} = 1.4327$) were dissolved in ether and reduced with hydrogen in the presence of platinum oxide[7] as catalyst. Reduction was complete in 2 hours. The ether extract was dried over anhydrous potassium carbonate. After removal of the ether the carbinol was distilled. It boiled at 66–67°, p = 20 mm. $n_D^{25}$ = 1.4198.

It analyzed as follows:

3.830 mg. substance: 10.210 mg. $CO_2$ and 4.810 mg. $H_2O$.
$C_7H_{16}O$.  Calculated.  C 72.41, H 13.79.
       Found.     " 72.65, " 14.05.

In a 1 dm. tube without solvent $\alpha_D^{25} = +5.50°$; $[\alpha]_D^{25} = +6.7°$. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{+0.82° \times 100}{1 \times 10.2} = +8.0°.$$

In ether the rotation was

$$[\alpha]_D^{25} = \frac{+1.00° \times 100}{1 \times 12.0}  +8.3°.$$

*Levo-Hexanal-(1)-ol-(2)* *(2-Hydroxycaproic Aldehyde).*—10 gm. of dextro-hepten-(1)-ol-(3) ($\alpha_D^{25} = +16.2°$ in a 1 dm. tube without solvent) were dissolved in 25 cc. of glacial acetic acid and ozonized in the usual way. The procedure for decomposition of the ozonide and the isolation of the hydroxyaldehyde was the same as described above. After standing in the refrigerator for several days the aldehyde crystallized.

It analyzed as follows:

3.390 mg. substance: 7.585 mg. $CO_2$ and 3.090 mg. $H_2O$.
$C_6H_{12}O_2$.  Calculated.  C 62.07, H 10.34.
        Found.     " 61.01, " 10.19.

[7] Voorhees, V., and Adams, R., *J. Am. Chem. Soc.*, **44**, 1397 (1922).

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{24} = \frac{-2.55° \times 100}{1 \times 8.85} = -28.8°.$$

*Relation of Ethylpropyl Carbinol to 2-Hydroxyvaleric Acid.*

*Dextro-Pentandiol-(1, 2) (1, 2-Dihydroxypentane).*[8]—12 gm. of levo-hexen-(1)-ol-(3) ($\alpha_D^{25} = -22.1°$ in a 1 dm. tube without solvent) were ozonized in glacial acetic acid in the usual way. The ozonide was decomposed in the usual manner. No attempt was made to isolate the hydroxyaldehyde. After removal of most of the ether the remaining syrup had $\alpha_D^{25} = +23.0°$ in a 1 dm. tube. The syrup was dissolved in 200 cc. of 50 per cent alcohol and reduced with sodium amalgam. The glycol was isolated by the usual procedure. It distilled at 88–90°, p = 2 mm. It analyzed as follows:

6 230 mg. substance: 13 315 mg. $CO_2$ and 6 225 mg. $H_2O$.
$C_6H_{12}O_2$. Calculated. C 57.69, H 11.53.
Found. " 58.28, " 11 18.

The rotation in absolute alcohol was

$$[\alpha]_D^{25} = \frac{+2.03° \times 100}{1 \times 12.1} = +16 8°.$$

The glycol was further identified by converting it into its phenylurethane. This melted at 108–110°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 5 7 cc 0 1 N HCl.
$C_{19}H_{22}O_4N_2$. Calculated. N 8 18 Found N 7 97.

The rotation in absolute alcohol was

$$[\alpha]_D^{25} = \frac{+1.18° \times 100}{2 \times 3.64} = +16 2°.$$

*Dextro-Hexanol-(3) (Ethylpropyl Carbinol).*—The relationship between levo-hexen-(1)-ol-(3) and dextro-ethylpropyl carbinol has been established.[9]

[8] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **77**, 555 (1928).
[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem*, **83**, 591 (1929).

# THE CORRELATION OF THE CONFIGURATIONS OF 2-, 3-, AND 4-SUBSTITUTED CHLORO- AND HYDROXY-ALIPHATIC ACIDS.

By P. A. LEVENE and H. L. HALLER.

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The work to be reported here is a continuation of the investigations which were the subject of three previous communications.[1,2]  It deals with the direction of the rotations of hydroxy acids and of the corresponding halogeno acids.  Levene[3] in cooperation with Mikeska and with Mori reached certain conclusions regarding these relationships by studying, on one hand, the behavior of the thiol and the corresponding sulfo acids and on the other, that of the hydroxy and halogeno acids.  The conclusions obtained in this manner were in harmony with the conclusions reached by Clough and by Holmberg, but were at variance with the conclusions of Freudenberg and of Kuhn and Wagner-Jauregg.  A detailed discussion of the disagreement was given in the first paper of this series.[1]

The following three groups of substances have been selected for our investigations.  (1) Monocarboxylic acids substituted in position 2; (2) monocarboxylic acids substituted in positions 3 or 4; and (3) substituted succinic acid.

Of the first group Levene and Mikeska and Levene, Mikeska, and Mori[3] have studied seven representatives, of the second Levene and Mikeska and Levene and Mori have studied three acids, and of the third, only one substance has been studied.  The conclu-

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 703 (1929); **82**, 185 (1929).

[3] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **70**, 365 (1926). Levene, P. A., Mori, T., and Mikeska, L. A., *J. Biol. Chem.*, **75**, 337 (1927). Levene, P. A., and Mori, T., *J. Biol. Chem.*, **78**, 1 (1928).

sions of these authors have been tested now by a second and independent method and the conclusions substantiated in regard to three substances; namely, (1) 2-chloropropionic acid, (2) 3-chlorobutyric acid, (3) chlorosuccinic acid.

It was felt, however, that the experience on a greater variety of compounds was desirable for a final decision of the disputed question. All of the analyzed 2-substituted thiol and sulfo acids behaved similarly in the respect which interested us; namely, the change in direction of rotation on passing from unionized state to the ionized was in the same direction in the thiol and in the sulfo acids. The observations have been extended to two additional 2-substituted acids, namely valeric and caproic acids.

In the case of the 2-substituted valeric acids the set of reactions leading to the solution of the problem of the relationship of the hydroxy and chloro acids was the following.

$$
\begin{array}{ccccc}
 & CH_3 & CH_2 & CH_2 & \\
 & | & \| & \| & \\
COOH & CH_2 & CH & CH & COOH \\
| & | & | & | & | \\
HCOH & \leftarrow HCOH & \leftarrow HCOH \rightarrow & HCCl & \rightarrow HCCl \\
| & | & | & | & | \\
C_3H_7 & C_3H_7 & C_3H_7 & C_3H_7 & C_3H_7 \\
\text{Levo.} & \text{Dextro.} & \text{Levo.} & \text{Dextro.} & \text{Levo.}
\end{array}
$$
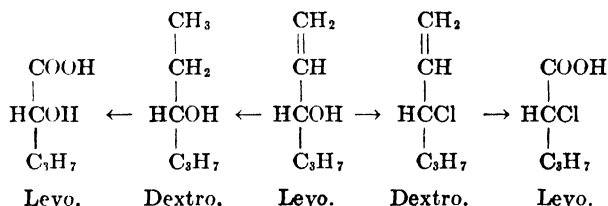
Thus, the conclusion is in complete harmony with that of Levene and Mikeska and of Levene, Mori, and Mikeska in regard to this pair of acids and in harmony with the conclusion regarding the 2-substituted chloropropionic acids reached by the second method.

The set of reactions and the conclusions in the case of the 2-substituted caproic acids were analogous.

$$
\begin{array}{ccccc}
 & CH_3 & CH_2 & CH_2 & \\
 & | & \| & \| & \\
COOH & CH_2 & CH & CH & COOH \\
| & | & | & | & | \\
HCOH & \leftarrow HCOH & \leftarrow HCOH \rightarrow & HCCl & \rightarrow HCCl \\
| & | & | & | & | \\
C_4H_9 & C_4H_9 & C_4H_9 & C_4H_9 & C_4H_9 \\
\text{Levo.} & \text{Dextro.} & \text{Levo.} & \text{Dextro.} & \text{Levo.}
\end{array}
$$
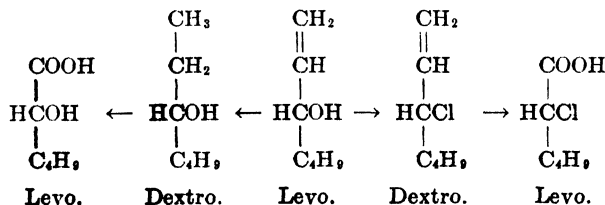
In this series of 2-substituted hydroxy and chloro acids the changes in the direction of rotation in passing from the unionized to the ionized state are identical.

The problem of the configurational relationships of the 3- and 4-substituted acids was more complicated. In the case of 3-thiolbutyric acid and the 3-sulfo acid derived from it the changes of the direction of rotation were different in each acid. Thus the dextro-thiolbutyric acid passing from the unionized to the ionized state changed its rotation to the left, whereas the corresponding sulfo acid changed the direction to the right. On the basis of this conduct, it was concluded that in the pair, 3-chloro- and 3-hydroxybutyric acids, the state of affairs was similar; namely, the levo-3-chlorobutyric acid, which on passing from the unionized to the ionized state changed its rotation to the right was co-related to the dextro-hydroxybutyric acid, which on passing from the unionized to the ionized state changed its rotation to the left. In the light of this exceptional position of these acids, Levene and Mori have investigated the conduct of the 3-, and of the 4-thiol-and sulfovaleric acids. These acids behaved normally and it was then concluded that the 3- and the 4-chloro- and hydroxyvaleric acids were configurationally related when the changes in the direction of rotation on passing to the ionized state were identical for the chloro and for the hydroxy acids. These observations on the valeric acids seemed to confirm the impression of the exceptional position of 3-substituted butyric acids. However, subjecting the problem of the configuration of 4-chlorovaleric acid to test by the new method, the result obtained was as given in the following set of figures.

$$
\begin{array}{cccc}
CH_3 & CH_3 & CH_2 & CH_2 \\
| & | & | & | \\
HCOH & HCOH & HCCl & HCCl \\
| & | & | & | \\
CH_2 & CH_2 & CH_2 & CH_2 \\
| & | & | & | \\
CH_2 \leftarrow & CH_2 \rightarrow & CH_2 \rightarrow & CH_2 \\
| & | & | & | \\
COOH & CH & CH & COOH \\
& \| & \| & \\
& CH_2 & CH_2 & \\
\text{Dextro.} & \text{Dextro.} & \text{Levo.} & \text{Levo.*}
\end{array}
$$
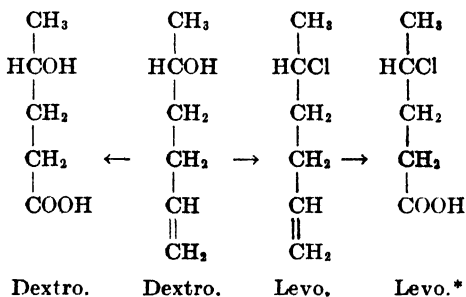
*$[M]_D$ free acid = $-$ 52.9° in 50 per cent alcohol.

$[M]_D$ Na salt = $-$ 43.3° in 50 per cent alcohol.

*In this case then the two methods lead to different conclusions. According to the second method the levo-4-chlorovaleric acid is*

*correlated to dextro-4-hydroxyvaleric acid in the same manner as levo-3-chlorobutyric acid is correlated to dextro-3-hydroxybutyric acid and in both cases the changes in direction of rotation on passing to the ionized state are in the opposite directions for the hydroxy and for the chloro acids. For the present we are inclined to regard the correlation based on the second method as the correct one.*

*On the basis of these observations, one will have to conclude that the rule of the change of direction of rotation in passing from the unionized to the ionized state is applicable as a guide for configurational relationships only in 2-substituted acids. In 3- and 4-substituted acids, the rule is applicable to the correlation of hydroxy acids among themselves, but is unreliable in application to acids in which the hydroxyl is substituted by groups or atoms of different polarity.*

It must be added that to date in our work the rotations were measured in the light of one wave-length only and at one temperature only. The rule of the change of direction of rotation may be found more comprehensive if the respective rotatory dispersions are measured. It is planned to do so in the near future.

We wish to add that we are conscious of the fact that the second method of correlating the configurational relationship of hydroxy and of halogeno acids would have gained much in weight if the unsaturated halides had been hydrogenated and if the hydrogenated products had been found identical with the halides prepared from the corresponding saturated carbinols. Our efforts to accomplish this end thus far have not been successful, although we have not abandoned the hope of accomplishing it at a future date.

### EXPERIMENTAL.

*Hexen-(1)-ol-(3) (Vinylpropyl Carbinol).*—This carbinol[4] was obtained on condensation of acrolein and propyl magnesium bromide in the usual manner.

*Resolution of Hexen-(1)-ol-(3).*[5]—The acid phthalate of the carbinol was prepared in the usual way[6] and then converted into the brucine salt in acetone. The resolution proceeded quite readily when 50 per cent alcohol was employed as a solvent. No

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[4] We are indebted to Mr. R. E. Marker for the preparation of the carbinol.

[5] Kenyon, J , and Snellgrove, D. R., *J. Chem. Soc.*, **127**, 1169 (1925).

[6] Levene, P. A , and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

attempt was made to obtain a carbinol of maximum optical activity. The usual procedure was employed for the isolation of the carbinol. It distilled at 133–134° at atmospheric pressure. It analyzed as follows:

4 750 mg substance: 12 600 mg $CO_2$ and 5.255 mg. $H_2O$.
No 1340. $C_6H_{12}O$. Calculated. C 72.0, H 12 0.
Found        " 72.33, " 12 38.

In a 1 dm. tube $\alpha_D^{25} = -19.40°$; $[\alpha]_D^{25} = -23.4°$.
In ether the rotation was

$$[\alpha]_D^2 = \frac{-\,1\,15° \times 100}{1 \times 9.87} = -\,11.7°.$$

In absolute alcohol it had the following rotation.

$$[\alpha]_D^2 = \frac{-\,2\,15° \times 100}{1 \times 9\,67} = -\,22\,2°.$$

*Dextro-3-Chlorohexene-(1).* —A solution of 19.5 gm. of levovinylpropyl carbinol (No. 1340) and 6 cc. of pyridine was dropped into 10 gm. of phosphorus trichloride. The reaction mixture was kept cold by immersion in an ice water bath and was constantly shaken. After the addition of the carbinol solution, the reaction mixture was allowed to stand for 1 hour and then the chloride was distilled under reduced pressure. p = 20 mm. The yield was 14 gm. It analyzed as follows:

0 1266 gm substance 0 1486 gm AgCl
No 1347 $C_6H_{11}Cl$ Calculated Cl 29 95 Found Cl 29 03

In a 1 dm. tube without solvent $\alpha_D^2 = +17.85°$.
In absolute alcohol the rotation was

$$[\alpha]_D^{25} = \frac{+\,2.13° \times 100}{1 \times 11\,86} = +\,18.0°.$$

In ether it had the following rotation.

$$[\alpha]_D^{25} = \frac{+\,1\,85° \times 100}{1 \times 9\,86} = +\,18.8°$$

*Levo-2-Chlorovaleric Acid.*—The chloride (No. 1347) obtained as described above was ozonized in chloroform solution in 3 gm. lots in the usual manner. The decomposition of the ozonide and the oxidation of the resulting aldehyde was effected by the procedure previously described for other unsaturated chloro derivatives.[7] The acid distilled at 80–84°, p = 1 mm. It analyzed as follows:

0 1190 gm. substance: 0.1324 gm. AgCl.
0.1480 gm. substance required 10.95 cc. 0.1 N NaOH. Calculated **10.84 cc.**
$C_5H_9O_2Cl$. Calculated. Cl 26.0. Found. Cl 27.52.

In ether it had the following rotation.

$$\text{\tiny ss} \quad \frac{-\ 0.79° \times 100}{1 \times 6.82} = -\ 11.6°.$$

0.431 gm. substance dissolved in 5 cc. of 50 per cent alcohol had the following rotation.

$$[\alpha]_D^{ss} = \frac{-\ 1.44° \times 100}{2 \times 8.62} = -\ 8.4°.$$

To 4.0 cc. of the above solution were added 2.6 cc. of 1.0 N NaOH; the rotation was observed immediately.

For the sodium salt,

$$[\alpha]_D^{ss} = \frac{-\ 0.36° \times 100}{2 \times 6.04} = -\ 3.0°.$$

*Dextro-Hexanol-(3)* *(Ethylpropyl Carbinol).*[8]—3.5 gm. of levo-vinylpropyl carbinol (No. 1340) were dissolved in a solution of 15 cc. of glacial acetic acid and 5 cc. of water and reduced with hydrogen in the presence of colloidal palladium as catalyst. After reduction had been completed the acid was neutralized with 25 per cent sodium hydroxide and the carbinol was extracted with ether. The ether extract was dried over anhydrous potassium carbonate and after removal of the ether, the carbinol was dis-

[7] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425, 703 (1929).
[8] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928).

tilled. It boiled at 130–132° at atmospheric pressure. It analyzed as follows:

4.225 mg. substance: 10.910 mg. $CO_2$ and 5.125 mg. $H_2O$.
$C_6H_{14}O$.  Calculated.  C 70.59, H 13.72.
            Found.      " 70.41, " 13.57.

In a 1 dm. tube without solvent $\alpha_D^{25} = +2.75°$.
In ether it had the following rotation:

$$[\alpha]_D^{30} = \frac{+\ 0.68° \times 100}{1 \times 13.1} = +\ 5.2°.$$

In absolute alcohol the rotation was

$$[\alpha]_D^{30} = \frac{+\ 0.45° \times 100}{1 \times 10.9} = +\ 4.1°.$$

*Dextro-3-Chloroheptene-(1)*.—This substance was prepared in the same manner as described for dextro-3-chlorohexene-(1). The hepten-(1)-ol-(3)[9] employed was levorotatory. $[\alpha]_D^{25} = -22.2°$ without solvent.

The chloride analyzed as follows:

0 1144 gm. substance: 0.1190 gm. AgCl.
   No. 1383.  $C_7H_{13}Cl$.  Calculated.  Cl 26.8.  Found.  Cl 25.73.

In a 1 dm. tube without solvent $\alpha_D^{25} = +14.60°$.
In ether it had the following rotation.

$$[\alpha]_D^{25} = \frac{+\ 0.98° \times 100}{1 \times 6.10} = +\ 16.1°.$$

In absolute alcohol the rotation was

$$[\alpha]_D^{25} = \frac{+\ 0.98° \times 100}{1 \times 6.06} = +\ 16.2°.$$

*Levo-2-Chlorocaproic Acid.*—The chloride (No. 1383) obtained as described above was oxidized to the chloro acid employing the procedure described for 2-chlorovaleric acid. The acid distilled at 80–95°, p = 1 mm. It analyzed as follows:

0.1084 gm. substance: 0 1044 gm. AgCl.
   $C_6H_{11}O_2Cl$.  Calculated.  Cl 23.58.  Found.  Cl 23.82.

[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 579 (1929).

In ether the rotation was

$$[\alpha]_D^{40} = \frac{-0.22° \times 100}{1 \times 10.3} = -2.1°.$$

In 50 per cent alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{-0.25° \times 100}{1 \times 14.2} = -1.8°.$$

*Dextro-Heptanol-(3) (Ethylbutyl Carbinol).*[9]—The relationship of levo-vinylbutyl carbinol to dextro-ethylbutyl carbinol has been described.

*Dextro-Hexen-(1)-ol-(5).*—The preparation[4] and the resolution of the carbinol was carried out in the same manner as previously described.[6]  The carbinol distilled at 138–139° at atmospheric pressure.  It analyzed as follows:

3 715 mg. substance: 9.865 mg. $CO_2$ and 4 035 mg. $H_2O$
No. 1209.  $C_6H_{12}O$.  Calculated.  C 72.0,  H 12.0
Found.      " 72 41,  " 12.15.

In a 1 dm. tube without solvent $\alpha_D^{22} = +12.25°$.
In absolute alcohol the rotation was

$$[\alpha]_D^{21} = \frac{+1.85° \times 100}{1 \times 12.3} = +15.0°.$$

In ether the rotation was

$$[\alpha]_D^{21} = \frac{+2.25° \times 100}{1 \times 12.8} = +17.6°.$$

*Dextro-Hexanol-(2) (Methylbutyl Carbinol).*[6]—5 gm. of hexen-(1)-ol-(5) (No. 1209) were reduced in ether solution in the usual manner.  The reduced carbinol distilled at 136–138° at atmospheric pressure.

It analyzed as follows:

3.095 mg. substance: 8.065 mg. $CO_2$ and 3.915 mg. $H_2O$.
No. 1379.  $C_6H_{14}O$.  Calculated.  C 70.58, H 13.72.
Found.      " 71.01,  " 14.15.

In a 1 dm. tube without solvent $\alpha_D^{22} = +8.25°$.

In ether the rotation was

$$[\alpha]_D^{25} = \frac{+ 1.50° \times 100}{1 \times 10.6} = + 14.1°.$$

In absolute alcohol the rotation was

$$[\alpha]_D^{25} = \frac{+ 1.70° \times 100}{1 \times 15.6} = + 10.9°.$$

*Levo-5-Chlorohexene-(1).*—50 gm. of dextro-hexen-(1)-ol-(5) (No. 1209) were dissolved in 100 cc. of dry ether and gradually added to a suspension of 115 gm. of phosphorus in 250 cc. of dry ether. The subsequent procedure was the same as that described for the chlorination of penten-(1)-ol-(4).[10] On distillation at atmospheric pressure a fraction was collected which distilled at 119–122°. This analyzed as follows:

0 1016 gm  substance: 0 1226 gm  AgCl
    No 1219   $C_6H_{11}Cl$   Calculated. Cl 29 95   Found. Cl 29 84.

In a 1 dm. tube without solvent $\alpha_D^{25} = -38.75°$.
In ether the rotation was

$$[\alpha]_D^{25} = \frac{- 3 50° \times 100}{1 \times 8 08} = - 43.3°.$$

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25} = \frac{- 4 30° \times 100}{1 \times 9 84} = - 43.7°.$$

*Levo-4-Chlorovaleric Acid.*—The chloride (No. 1219) obtained as described above was ozonized in chloroform solution. The procedure was the same as described under 2-chlorovaleric acid. The acid distilled at 95–100°, p = 1 mm.
It analyzed as follows:

0 1276 gm. substance: 0.1452 gm. AgCl.
        $C_5H_9O_2Cl$. Calculated. Cl 26.0. Found. Cl 28.15.
    0.1855 gm. substance required 12 80 cc. 0.1 N NaOH. Calculated. 13.6 cc.

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[10] Levene, P. A , and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

In ether the rotation was

$$[\alpha]_\mathrm{D}^{22} = \frac{-9.00° \times 100}{2 \times 11.0} = -40.9°.$$

0.397 gm. substance was dissolved in 5 cc. of 50 per cent alcohol. For the free acid

$$[\alpha]_\mathrm{D}^{22} = \frac{-6.17° \times 100}{2 \times 7.94} = -38.9°.$$

To 3 cc. of the above solution were added 1.75 cc. of 1.0 N NaOH and 0.6 cc. of absolute alcohol. For the sodium salt

$$[\alpha]_\mathrm{D}^{22} = \frac{-2.83° \times 100}{2 \times 5.17} = -27.4°.$$

# THE INFLUENCE OF FORMALIN FIXATION ON THE LIPOIDS OF THE CENTRAL NERVOUS SYSTEM.

By ARTHUR WEIL.

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During an investigation on lipoid changes of the central nervous tissues under pathological conditions, the necessity for a reliable quantitative method which would allow a differentiation of the manifold lipoids of the tissues arose. At the same time the problem had to be solved whether it was possible to utilize formalin-fixed tissue instead of fresh material.

The enthusiasm which followed the publications of Aschoff and his pupil Kawamura (1) on the differential staining of the lipoids has gradually subsided. It even gave way to a severe criticism of the many histological methods which had been devised based partially on these investigations (Smith, Ciaccio, Dietrich, etc.). Critical observers pointed out that one is not safe in applying test-tube staining methods of isolated lipoids or of their mixtures to the staining of sections of tissue. Kutschera-Aichbergen (2) found that after extraction with warm acetone the extracted sections of suprarenal glands, of brain, and of sclerotic arteries did not stain with the above methods. Besides the neutral fats and cholesterol, acetone also removes a small part of the phosphorus-containing lipids.[1] Kaufmann and Lehmann (3) demonstrated recently that the Ciaccio method depends on the presence of oleic acid and that it is not specific for any given lipid. Shapiro (4) reached conclusions similar to those of Kutschera-Aichbergen.

Aside from the present histological methods for the differentiation of the lipoids there remain the quantitative chemical methods for fractional extraction (Fraenkel, MacLean (5)). Many of the figures on the lipoid content of human tissues which are pub-

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[1] Lipoids = total of fatty substances = neutral fats + cholesterol and its esters + lipids (phosphatides and cerebrosides).

lished in the text-books have been taken from analytical work on formalin-fixed material. This applies especially to the work of Smith and Mair (6) on the development of the brain of growing animals and man, on the comparative studies of gray and white matter, and on the lipoid changes of the nervous system in disease. No attempt seems to have been made to study the influence of the fixing agents on the final results of those methods. It seems to be generally recognized that formalin, the fixing agent which at present is mostly applied for the conservation of nervous tissue, is an excellent preservative for the lipoids (Romeis (7), Lee (8)). Fresh, unfixed material after extraction with acetone does not stain with Sudan III, the Smith, and the Ciaccio methods. Kutschera-Aichbergen, however, demonstrated that formalin-fixed sections of different tissues gave positive staining reactions with these methods after the different steps of extraction with acetone, alcohol, and ether. Spatz (9) has shown that formalin which had been neutralized and which had served for fixation of brains reacted acid after some time. The acidity increased with progressive preservation, while formalin alone without tissue did not undergo similar changes. He thought that the combination of formaldehyde with the amino acids of the proteins and the resulting acid methylene substances were the reason for the constantly increasing acid reaction. The method of Sörensen for the formol titration of amino acids is based on this chemical reaction. Blum who first recommended formaldehyde solutions for fixing purposes has also studied the reaction with the proteins (10).

The aim of the present investigation was to study the behavior of the lipoids of the central nervous system following formalin fixation. The method of fractional extraction which was applied is based on the work of H. and I. S. MacLean (5). The different steps of the extraction may be outlined in the accompanying scheme. A more detailed description has been given elsewhere (11).

1. The fresh substance is extracted with acetone at room temperature.

2. The dried tissue is extracted with absolute alcohol in a Soxhlet apparatus.

3. The extract is concentrated and excess of acetone added.

4. The precipitate is extracted with cold alcohol.

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                    ┌─────────────────────────┬─────────────────────────┐
            Soluble part;                            Insoluble part;
            lecithin chiefly                      cephalin chiefly also
                    │                         sphingomyelin and galactolipids
                    │                                     │
            Treated with ether                    Treated with ether
        ┌───────────┴───────────┐         ┌───────────────┴───────────────┐
   Solution contains   White remainder   Solution contains   White remainder con
      lecithin          consists of traces    cephalin          tains  sphingomyelir
                        of sphingomyelin                         and galactolipids
                        and galactolipids                              │
                                                             Treated with pyridine
                                                        ┌────────────────┬────────────────┐
                                                    Soluble part,        Insoluble part,
                                                    galactolipids        sphingomyelin
                                                      chiefly               chiefly
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I am obliged to Dr. J. P. Simonds and Dr. W. W. Brandes for the four human brains which were used for this study and all of which were removed within 5 to 10 hours after death. The central nervous system of these cases did not show anatomical signs of disease. One brain was obtained 6 hours after death from an accident and served as control. In the accompanying tabular matter average figures are given because the present investigation does not attempt to establish standard figures for brains of different age and sex. The intention was to demonstrate the changes of the different lipoid fractions during the progressing process of formalin fixation.

First the amount of phosphorus which was found in the fixing fluid after different stages of fixation was determined. It was found that with 2 liters of 10 per cent formalin (4 per cent solution of formaldehyde) per 1000 gm. of brain 110 mg. of phosphorus per 1000 gm. of fresh brain had passed into the fixing fluid after 24 hours. This formalin was renewed after 24 hours and phosphorus analyses of this second formalin (Neumann's method) were were made at different intervals with the following results.

| Days of fixation | 22 | 27 | 38 | 63 | 93 | 109 |
|---|---|---|---|---|---|---|
| Mg. P per 1000 gm. fresh brain | 340 | 378 | 400 | 429 | 476 | 532 |

TABLE I.

*Fractional Extraction of Fresh and Formalin-Fixed Nervous Tissue in Per Cent of Dry Substance. Water in Per Cent of Fresh Tissue.*

Averages from the central nervous systems of six cats (fresh), five cats (formalin), and three human brains.

| | Cats. | | | | | Human brains. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Brains. | | Spinal cords. | | | Gray matter. | | | White matter. | | |
| Days...................... | 0 | 22 | 0 | 22 | 92 | 0 | 22 | 102 | 0 | 22 | 102 |
| Water in per cent of fresh tissue.......... | 75.2 | 77.2 | 67.8 | 68.7 | 71.1 | 82.0 | 85.3 | 84.3 | 68.6 | 72.7 | 73.1 |
| I. Acetone extract.. | 10.9 | 12.1 | 17.3 | 20.3 | 22.6 | 15.4 | 19.8 | 16.3 | 21.3 | 23.7 | 20.0 |
| II. Alcohol    "  .. | 45.5 | 36.1 | 55.3 | 43.8 | 42.8 | 31.0 | 21.3 | 20.4 | 47.6 | 40.0 | 40.4 |
| Total      "  .. | 55.5 | 48.2 | 72.6 | 64.1 | 65.4 | 46.4 | 41.1 | 36.7 | 68.9 | 63.7 | 60.4 |
| Precipitate ........... | 18.0 | 15.7 | 28.0 | 25.3 | 22.5 | 12.2 | 7.8 | 6.9 | 30.6 | 22.1 | 19.2 |
| III. Alcohol-soluble... | 5.0 | 4.9 | 7.8 | 8.4 | 9.4 | 2.7 | 1.8 | 2.5 | 5.2 | 9.1 | 6.9 |
| IV. Ether-soluble..... | 6.9 | 4.3 | 9.8 | 7.3 | 6.5 | 5.6 | 2.8 | 1.7 | 11.3 | 5.5 | 3.2 |
| V. Pyridine-soluble.. | 2.4 | 3.1 | 4.6 | 5.5 | 4.6 | 2.0 | 2.1 | 2.0 | 9.9 | 6.1 | 7.6 |
| VI. Pyridine-insoluble | 3.7 | 3.4 | 6.1 | 4.1 | 2.0 | 1.9 | 1.1 | 0.7 | 4.2 | 1.4 | 1.5 |
| Rest of extraction..... | 44.5 | 51.8 | 27.4 | 35.9 | 34.6 | 53.6 | 58.9 | 63.3 | 31.1 | 36.3 | 39.6 |

TABLE II.

*Distribution of Phosphorus in Different Extracts. Averages of Three Human Brains.*

| | Gm. P per 1000 gm. dry substance. | | | | | | Gm. P per 1000 gm. fresh substance. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gray matter. | | | White matter. | | | Gray matter. | | | White matter. | | |
| Days.......... | 0 | 22 | 102 | 0 | 22 | 102 | 0 | 22 | 102 | 0 | 22 | 102 |
| Acetone extract..... | 1.89 | 2.75 | 2.56 | 1.14 | 1.81 | 1.37 | 0.34 | 0.40 | 0.40 | 0.36 | 0.49 | 0.37 |
| Alcohol extract..... | 7.52 | 6.87 | 6.46 | 9.20 | 7.63 | 7.23 | 1.36 | 1.01 | 1.01 | 2.89 | 2.08 | 1.94 |
| Total    extract..... | 9.41 | 8.62 | 9.02 | 10.34 | 9.44 | 8.60 | 1.70 | 1.51 | 1.51 | 3.25 | 2.57 | 2.31 |
| Rest of extract..... | 4.72 | 4.28 | 5.03 | 2.82 | 3.25 | 3.24 | 0.85 | 0.63 | 0.79 | 0.88 | 0.89 | 0.87 |
| Total phosphorus... | 14.13 | 14.19 | 14.05 | 13.16 | 12.69 | 11.84 | 2.55 | 2.14 | 2.30 | 4.13 | 3.46 | 3.18 |

In order to determine whether this increase in phosphorus was due to a gradual extraction of formalin-soluble phosphorus-containing substances from the brain, to an incomplete fixation with continuing autolysis, or to a direct action of the formalin on the brain the following experiment was made. Fresh brain tissue was ground in a mortar and for 3 hours extracted in a Soxhlet apparatus with boiling water. By this process all fermentative action was interrupted and autolysis prevented. Furthermore all the water-soluble phosphorus-containing substances were extracted. This thoroughly extracted and denatured brain substance was preserved in 10 per cent formalin. After 22 days 256 mg. of P per 1000 gm. of fresh brain were found, after 63 days this amount had increased to 453 mg. per 1000 gm. of brain. This result demonstrates that the third assumption which was stated above is the reason for the increase of phosphorus in the fixing fluid.

To control the results obtained from human material, brains and spinal cords of cats were removed immediately after death and preserved in 10 per cent formalin. After 22 and 92 days respectively they were treated with the same method of fractional extraction that had been applied to the human fresh or formalin-fixed material.

Tables I and II give the values obtained.

<center>DISCUSSION.</center>

Both the material obtained from human brains and the central nervous system of cats showed similar changes under the influence of formalin fixation. The increase in water, with swelling, in formalin solutions has been frequently noticed and reported (12). The influence of the lipoid content on the swelling of the brain has been studied earlier (13). The acetone extracts containing neutral fats, cholesterol, and its esters, and small amounts of phosphatides are not changed except in the spinal cords of cats. In the human material there seems to be an increase of the phosphorus-containing substances which are dissolved by acetone after 22 days; after 102 days this amount is the same as in the non-fixed material. The alcohol extracts containing the lipids are considerably diminished and consequently the amount of phorphorus-containing lipids is in the human material reduced by about 30 per cent at the end of 102 days. At the same time

the amount of lipids which can be precipitated from the alcohol extract by acetone (precipitate in Table I) has decreased correspondingly. In analyzing the different fractions of this precipitate it will be found, that the alcohol-soluble part (containing mainly lecithin) has not changed or is even increased. The same is true of the pyridine-soluble part (containing mainly the galactolipids or cerebrosides). On the other hand the ether-soluble part (containing mainly cephalin) and the part which is not soluble in alcohol, ether, or pyridine (mainly sphingomyelin) are reduced to one-third of the original value.

An analysis of Table II demonstrates that the total content of phosphorus calculated for the fresh substance is diminished by 10 per cent in the gray and by 23 per cent in the white matter of the brain. The amount of phosphorus in mg. which was dissolved by the formalin is calculated from these values as 551 mg. per 1000 gm. of brain. In order to be able to carry out the calculation of the phosphorus content of the whole brain, one has to know the relative part of white and gray substance. This relationship can be established by weighing the separated gray and white substance of a brain, which is a task difficult to perform. A second way is to determine this relationship from the water content of gray matter, white matter and the total brain. Two formulas have to be applied: (1) $a x + b y = 100 c$; and (2) $x + y = 100$ ($x$ = white matter, $y$ = gray matter in per cent of total brain; $a$, $b$, and $c$ = water content of white matter, gray matter, and total brain respectively in per cent of fresh substance). From the averages of a large number of normal brains the relation of 43 per cent white matter: 57 per cent gray matter has been established (14). The number of 551 mg. of phosphorus per 1000 gm. of brain which was thus calculated corresponds very closely to the amount (532 mg. of P) actually found in the formalin after 102 days.

From these data one may reconstruct the action of formaldehyde solution on the lipoids of the central nervous system as follows. Preservation in formalin does not fix the lipoids. After thorough extraction with alcohol in the Soxhlet apparatus the amount of phosphorus which remains in the residues is the same in fresh tissues as in formalin-fixed tissues. The amount of acetone-soluble substances (neutral fats, cholesterol, and its esters) also remains approximately the same. On the other hand the phos-

phatides which can be extracted with alcohol in the Soxhlet apparatus have been hydrolyzed under the influence of the formaldehyde. The phosphoric acid component is split off and is finally found in a combination which is water-soluble. This hydrolyzing process seems to affect mainly the cephalin and the sphingomyelin fractions. Between the beginning of formalin preservation and this final stage there seems to be a stage in which first intermediary products are formed. These are not yet soluble in water but easily soluble in acetone and alcohol. The galactolipids (cerebrosides) which do not contain phosphoric acid are preserved.

Many facts which by microscopical study are already empirically known to the pathologist may be explained on the basis of the above findings.

1. Galactolipids (cerebrosides) form a considerable part of the white matter. The white matter of the brain and the spinal cord is mainly composed of the conductive elements of the nervous tissue, i.e., axons and their myelin sheaths, while the gray matter contains mainly the ganglion cells. The other fractions of the lipids are twice as large in the white matter and in the spinal cord as in the gray matter. The amount of galactolipids, however, is 5 times as large. The galactolipids are not soluble in alcohol and ether at room temperature, but are soluble in pyridine. This may explain the favorable effect of the staining of the axons after pyridine treatment in the methods of Cajal, Ranson, and Bielschowsky. The removal of the galactolipids makes the subsequent penetration with silver nitrate easier.

2. Metachromatic staining bodies which also stain with mucicarmine have been described in formalin-fixed nervous tissue, which had been treated with alcohol (Buscaino's "grape-like bodies", "mucocitic degeneration of the neuroglia" of Grynfeltt and Pelissier, "mucine-like bodies" of Lhermitte, Kraus, and Bertillon (15)). Bailey and Schaltenbrand showed similar staining reactions in swollen oligodendroglia and assumed that the bodies mentioned above may originate from the decomposition of these diseased glia cells (16). Mucicarmine not only stains mucine, i.e. combinations of proteins with glucosamine, but also other substances, e.g., galactolipids. It does not stain the rest of the lipids, as may easily be demonstrated by the following experiments. Chloroform solutions of the different fractions which were

obtained by the extraction of brain tissue (Fractions I, III, IV, V, and VI of Table I) are spread on glass slides and allowed to dry. They are simultaneously stained for 2 minutes in a 1 per cent solution of mucicarmine and then rinsed in water. Only the slide with Fraction V (containing galactolipids) is stained red. Toluidine blue, however, stains all the different fractions with the exception of Fraction I (neutral fats and cholesterol) blue; Fraction III is stained lighter than the three others. Treatment with 95 per cent alcohol for a sufficient length of time will produce a distinct shade of pink color in Fraction V, while Fractions III, IV, and VI stain more blue.

The mucine bodies are not soluble in alcohol and ether at room temperature but are soluble in chloroform. They cannot be removed by ammonia or dilute acids, but they disappear in hot water at 60°. Galactolipids have similar physical qualities; besides they are relatively increased in the white matter after formalin treatment. By treatment of formalin tissue with alcohol the remainder of the phosphatides which was not hydrolyzed is removed (sphingomyelin having for the greater part been previously destroyed), while galactolipids remain. Ferraro states that he found grape-like areas in the white matter of frozen sections from formaldehyde-fixed material only after treatment with alcohol.

Considering all these different facts one might advance the hypothesis that the staining and other physical qualities of these different areas or bodies are due to the presence of free galactolipids (cerebrosides). Their seemingly frequent appearance in the white matter of diseased brains fixed in formalin may be explained by the assumption of lipid changes *intra vitam* which favored the following liberation of the galactolipids from the rest of the lipids by formalin and alcohol treatment. Preliminary experiments seem to indicate that the appearance of phosphorus in the fixing fluid occurs much more rapidly in pathologic brains than in normal ones.

### CONCLUSIONS.

1. The present opinion that formaldehyde solutions are a fixative for the preservation of lipoids should be revised.

2. In a 10 per cent solution of formalin (4 per cent solution of

formaldehyde) the phosphatides are hydrolyzed and the liberated phosphoric acid is found in a water-soluble composition in the fixing fluid. This process of decomposition proceeded gradually and was still found after 90 days fixation.

3. Cholesterol and galactolipids (cerebrosides) are not appreciably changed. Consequently the resulting mixture of lipids after formalin fixation contains more galactolipids than the original tissue.

4. The preservation and relative increase of galactolipids in formalin-fixed tissue have been utilized to explain some empirically known histological facts; namely, the effect of pyridine in silver staining methods of nerve fibers and the staining and physical qualities of areas of so called "mucoid degeneration" which are found in formalin material after alcohol treatment.

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# A MANOMETRIC METHOD FOR THE DETERMINATION OF GAS IN FERMENTATIONS.

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The method previously described[1] for determining carbon dioxide during fermentation is specific for that substance and may not be used for other gaseous products. The method has also the disadvantage that volatile substances such as toluene or acetaldehyde are removed from the fermenting mixtures and their use is thus excluded. To obviate these difficulties a new apparatus has been devised, based on Slator's[2] method of measuring the change in pressure at constant volume. In addition to these improvements a shaking device has been designed which greatly facilitates the introduction and removal of the tubes. A year's experience with the new apparatus has shown it to be so satisfactory that a description seems warranted.

The fermentations are performed in tubes which are immersed in a small thermostat and are actively shaken except when readings are being made. The tubes are partially evacuated at the start and the increase in pressure is measured at constant volume on a modified Van Slyke type manometer.[3]

The manometer is shown in Fig. 1. In place of the usual leveling bulb, a closed mercury reservoir has been substituted. The reservoir consists of a stoppered Erlenmeyer flask into which two side tubes have been sealed. The lower of these is connected by heavy rubber tubing to the bottom outlet of the manometer while the upper side tube, which is well above the mercury level, is attached to a 3-way stop-cock, *A*. One branch of this stop-cock

[1] Raymond, A. L , and Winegarden, H. M., *J. Biol. Chem.*, **74,** 189 (1927).

[2] Slator, A., *J. Chem Soc.*, **89,** 128 (1906).

[3] Van Slyke, D. D , *J. Biol. Chem.*, **73,** 121 (1927).

is open to the atmosphere and the other is connected to a 2 liter bottle in which a considerably diminished pressure is constantly maintained by a water aspirator. By turning the stop-cock one way or the other, the pressure above the mercury may be made
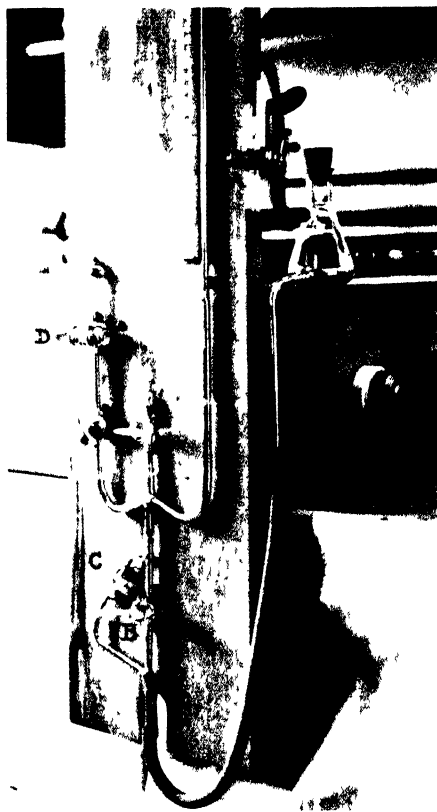


FIG. 1. Fermentation apparatus. Front view showing mercury reservoir and manometer connections.

atmospheric or less as desired. This is equivalent to raising or lowering the usual leveling bulb but is both faster and more convenient.

In order further to speed up the determinations, the bottom stop-cock, *B,* of the regular Van Slyke manometer is by-passed

with a branch which carries another smaller stop-cock, $C$, and, just below it, a very fine constriction. The constriction is so chosen that the mercury flows through it only very slowly. The rough adjustments are performed with Stop-cock $B$ while the final settings are made by using Stop-cock $C$. Although these changes may appear to complicate the manipulation, actually they result in a considerable time-saving.
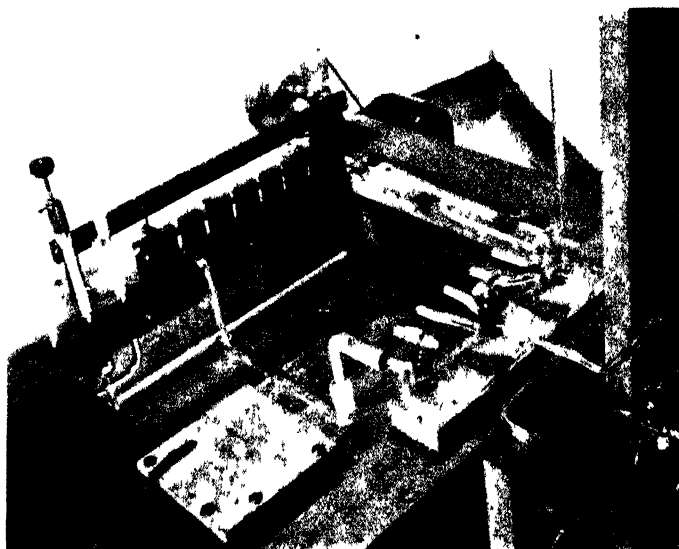


Fig 2 Fermentation apparatus    Top view showing shaker and method of connecting tubes

In Fig. 2 are shown the thermostat and shaking device and the manifold for connecting the tubes to the manometer. The manifold is of capillary tubing and consists of three 3-way stop-cocks so arranged that any one of the six tubes may be selected.[4] Sealed to the manifold there is another 3-way stop-cock, $D$, one branch of which leads to the manometer while the other is open. Through this open branch the tubes may be evacuated at the beginning or during a run, as desired. A vacuum source is provided for this

[4] The apparatus was arbitrarily designed for six tubes.

purpose by placing another outlet from the vacuum reservoir near at hand (the 2-way Stop-cock $E$ in Fig. 2).

The tubes which we use are of the two types shown in Fig. 3. The lower is a closed tube, sealed at one end, while the upper is designed to permit the introduction of solutions through an inlet equipped with a stop-cock. The support arms are furnished with copper sleeves into which are soldered small brass rods bent to a right angle. The sleeves are attached to the glass arms with sealing wax or with De Khotinsky cement.
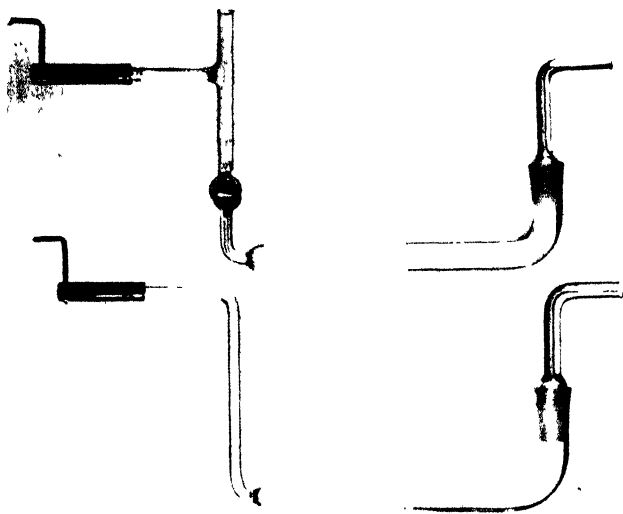


Fig 3   Fermentation tubes. The lower is a closed tube, sealed at one end; the upper has an inlet equipped with a stop-cock.

Referring again to Fig. 2, the method of operation is seen. The tubes are attached at one end to the manifold with rubber connections, and at the other end rest in slots cut in a Bakelite support. The brass arms fit into corresponding slots in another Bakelite bar which is oscillated horizontally by the motor-driven eccentric seen at the right. The eccentricity is small, the pin being set only 2.5 mm. off center, and this permits a shaking of high frequency and small amplitude. The motor has a variable series resistance to afford speed adjustment.

At high speeds the shaking arms tend to rise out of the slots and to avoid this a hinged bar is attached to the back of the Bakelite support. To the bar are soldered thin flat strips of spring steel which press lightly on the copper sleeves and hold the arms in the slots.

The thermostat needs little comment. A copper tank, placed in a wooden box and packed with hair-felt to avoid excessive heat loss was found satisfactory. A toluene-mercury thermoregulator operates a 60 or 100 watt lamp through a relay. The regulator and lamp support are seen at the left in Fig. 2.

For satisfactory operation of the apparatus it is necessary to have perfect vacuum-tight rubber connections between the tubes and manifold. After trying several grades of tubing and a number of lubricants without success, it was finally found that heavy red stethescope tubing, impregnated with vaseline, made very satisfactory joints. In preparing the tubing it is cut to short lengths, placed in a wide-mouth flask, covered with melted vaseline, and heated under reduced pressure at about 65° until nearly gas-free. After pouring off the liquid vaseline and wiping off the excess, the pieces are stored in a stoppered bottle until needed. Just before use they are lightly lubricated with vaseline and will then slip easily over the glass parts. The glass-rubber attachment is so firm, however, that it is necessary to cut the connections off with a sharp knife or razor blade at the end of a run.

For the glass joints, any good grade of lubricant may be used; in our own case we employ Lubriseal (A. H. Thomas) for the stop-cocks and Vacuum Wax (11028 Central Scientific Company) for sealing the ground joints of the fermentation tubes.

The mercury must not adhere to the capillary manifold or the readings are incorrect. Chromic acid cleaning mixture may be used, followed by distilled water and by acetone, and the latter then removed by sucking clean dry air through the manifold till dry.

The motor used for the shaker is a Westinghouse sewing machine motor, but for satisfactory operation at low speeds it is essential that the commutator and brushes be kept scrupulously clean. To this end it is desirable to use little or no lubricant at the commutator end of the motor.

The technique of operation as now used may be best illustrated by describing a typical run. The thermostat is brought to tem-

perature by using a bent glass rod in place of one of the tubes as a stirrer to attain uniform temperature throughout the bath. The tube stoppers are attached to the manifold with the vaselined rubber connections as described. The ground joints of the fermentation tubes are greased with Vacuum Wax (not too heavily) and the tubes are then filled with the fermentation mixtures. The spring-bar is unscrewed and the tubes are placed in position one by one, the ground joints being well seated.

The spring-bar is then put back in place. Stop-cock $D$ is turned to the open branch which is then attached to the vacuum reservoir. The manifold stop-cocks are turned so as to evacuate each fermentation tube in turn. The reservoir pressure should not be too low, or it may cause the mixtures to froth. Stop-cock $D$ is next shut (as in Fig. 2) and the ground joints of the fermentation tubes are reseated to assure perfect seals. The shaker may then be started, its speed being such as to break up the liquid surface as much as possible. It will be found that several "synchronization speeds" exist at which the liquid remains relatively quiet and these are to be avoided.

For making pressure readings the following procedure has been found satisfactory. The shaker is stopped and with Stop-cock $D$ and all the manifold stop-cocks shut, Stop-cock $A$ is turned to connect with the vacuum source. Stop-cock $B$ is carefully opened until the pressure recorded on the manometer is a little less than that existing in the tube which is to be read. Stop-cock $B$ is shut, Stop-cock $A$ is turned to allow air to enter the reservoir, the manifold stop-cock is opened to the particular tube desired, and Stop-cock $D$ is turned to connect the manifold with the manometer. The mercury flows into the manometer but the bulb will allow for all reasonable differences in pressure. Stop-cock $B$ is now cautiously opened and the mercury rises and enters the manifold. Stop-cock $B$ is shut and Stop-cock $C$ is operated until the mercury approaches to within 4 to 5 mm. of the manifold stop-cock. Stop-cocks $C$ and $D$ and the manifold stop-cock are then shut in this sequence and the pressure is read on the manometer. This is repeated for each tube.

One familiar with the apparatus can read six tubes in about 2 minutes (except for the first set of readings) and during this time most of the gas (if $CO_2$) which is formed will remain dissolved in the

liquid if the shaker is not running. The time of reading may therefore be taken at the moment of stopping the shaker and will be the same for all six tubes.

One further word may be added about the manometer tube itself. This is a simple tube, completely filled with mercury and then shut off with a stop-cock which is mercury-sealed. In the Van Slyke model the tube is graduated, but for our purposes such high accuracy was not required and a meter stick alongside was found quite satisfactory. It may be mentioned that the tube when graduated is scaled in millimeters and not in volume.

For calculating the volume of gas evolved the following formula obtains

$$\Delta V \quad \left[ \frac{(P_2 - p_2)V_2}{T_2} \quad \frac{(P_1 - p_1)V_1}{} \right]$$

where $\Delta V$ is the volume of gas evolved;
$\qquad P$ is the manometer reading;
$\qquad p$ is the vapor pressure of the fermenting mixture;
$\qquad V$ is the volume of the gas space;
$\qquad T$ is the absolute temperature of the gas;
$\qquad C$ is a conversion constant;
$\qquad$ and where the subscripts refer to a first and second reading.

However when the temperature is kept constant and there is no significant change in the liquid, then the formula reduces to

$$\Delta V = (P_2 - P_1) \frac{V}{T} C$$

If $P_1$ and $P_2$ are expressed in mm., $V$ in cc., and $\Delta V$ in cc. of gas under standard conditions (760 mm. and 0°) and $T$ is the absolute temperature, then

$$C = 0.359$$

$V$ may be calculated with sufficient accuracy by subtracting the volume of the fermenting mixture from the total volume of the tube and stopper, plus the added value of the gas space in the manifold and manifold stop-cock. This last correction is small; only about 0.2 cc. in the particular machines which we are using, and is practically the same for each connection. For convenience, we number each tube and its corresponding stopper and determine the volume of the two by weighing, filled and empty. To

this is added the 0.2 cc. correction for the gas space of the manifold, and this corrected volume is tabulated against the tube number. These volumes are used in making the calculations.

With tubes of the size illustrated and containing about 4 cc. of liquid, an increase in pressure of 1 mm. corresponds to the production of about 0.02 cc. of gas (under standard conditions) or about $10^{-1}$ millimols. The sensitivity may be increased if desired, by using smaller tubes or by filling them with more liquid.

### SUMMARY.

An apparatus is illustrated for determining the gas production in fermenting mixtures. It is based on Slator's method of measuring the increase in pressure at constant volume but has been designed for easy and rapid determination of several simultaneous samples.

The details of construction and manipulation are described.

# SYNTHETIC HEXOSEPHOSPHATES AND THEIR PHENYLHYDRAZINE DERIVATIVES.

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The carbohydrate esters of phosphoric acid have acquired considerable significance during recent years due to the fact that they are involved in many biological processes of carbohydrate utilization. One of the ways leading to the understanding of their biological significance is the study of the behavior of isomeric phosphoric esters, differing from the naturally occurring ones in the allocation of the phosphate group.

A number of such isomers have been described in the literature but their preparation was associated with many difficulties. As we had occasion to employ several of these esters, we developed improved procedures for their preparation which have simplified the operations and increased the yields. The general methods employed were those of Levene and Meyer,[1] Komatsu and Nodzu,[2] and Nodzu[3] and were of two types. In one, the hexoses were directly phosphorylated, leading probably to a mixture of isomers. In the other, the diacetone hexoses were phosphorylated and the acetone groups were subsequently removed by mild acid hydrolysis, giving products which were presumably single isomers. The present paper describes in detail the procedures which were evolved for these syntheses, as well as for the preparation of the diacetone hexoses.

A further matter of interest in connection with the synthetic esters is their behavior with phenylhydrazine, for derivatives of

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **53**, 431 (1922).

[2] Komatsu, S., and Nodzu, R., *Mem. Coll. Sc., Kyoto Imp. Univ.*, **7**, series A, 377 (1924).

[3] Nodzu, R., *J. Biochem.*, Japan, **6**, 31 (1926).

this type help to identify the esters and may also aid in the allocation of the phosphate group in those cases where the structure has not been fully established.

The ester prepared by direct phosphorylation of glucose was the least interesting as it gave no crystalline hydrazone or osazone.

The glucose monophosphate prepared through the diacetone glucose likewise yielded no hydrazone. However, on being heated with phenylhydrazine the phosphate group was eliminated, giving a phosphorus-free osazone. Interestingly, this proved to be an anhydrohexosazone apparently identical with that described by Levene and Sobotka,[4] and which we had previously[5] obtained under similar conditions from the fructose phosphate prepared through α-diacetone fructose. The relatively great stability[3] of the phosphate group in the glucose derivative seems to indicate that a phosphorosazone is first formed, rendering the phosphate less stable. Hydrolysis follows, accompanied by Walden inversion as in the case of the fructose derivative. Since each of the parent diacetone hexoses mentioned above had position 3 free, these two esters should be 3-glucose and 3-fructose phosphate. This reaction of osazone formation with ring closure and Walden inversion may prove common to all hexoses phosphorylated in position 3.

The fructose derivatives likewise gave very interesting results. The ester prepared through β-diacetone fructose gave a diphenylhydrazine phosphohydrazone; while on forming the osazone, the phosphorus was eliminated and a good yield of glucosazone was obtained. As Ohle[3] has established the position of the free hydroxyl in this diacetone to be carbon atom 1, the phosphate should be 1-fructose phosphate. The formation of both the phosphohydrazone and glucosazone is exactly analogous to the behavior of the Harden diphosphate and lends further support to the view that the latter is a fructose derivative with one phosphate attached to carbon atom 1.

The ester- prepared by direct phosphorylation of fructose also gave a fair amount of glucosazone, again suggesting phosphoryla-

[4] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **71**, 181 (1926–27).

[5] Levene, P. A., Raymond, A. L., and Walti, A., *J. Biol. Chem.*, **82**, 191 (1929)

[6] Ohle, H., *Ber. chem. Ges.*, **58**, 2577 (1925). Ohle, H., and Berend, G., *Ber. chem. Ges.*, **60**, 1159 (1927).

tion on carbon atom 1. This result was surprising as the more usual view is that substitution takes place on carbon atom 6 and we had expected an osazone identical with that from the Neuberg ester (which is 6-fructose monophosphate[7]). In this connection it is interesting to note that the monophosphate from fructose shows a behavior quite different from that of the Neuberg ester when submitted to enzymatic fermentation[8] thus confirming the difference in structure. The phosphorylation on atom 6 is perhaps prevented by the formation of the normal $<2, 6>$ lactal ring in the free fructose.

The results with the phosphate prepared through the $\alpha$-diacetone have been described in part elsewhere.[5] This ester gave a phenylhydrazine phosphohydrazone in the cold, while on boiling, the phosphate was eliminated with the formation of an osazone. Unexpectedly, however, the analysis indicated an anhydrohexosazone which proved, as mentioned above, to be similar to the 3, 6-anhydrohexosazone of Levene and Sobotka.[4]

## EXPERIMENTAL.

### I. Preparation of Phosphoric Esters.

#### A. Phosphorylation of Glucose.

Dissolve 15 gm. of powdered anhydrous glucose in 100 cc. of boiling anhydrous pyridine in a 6 × 20 cm. Pyrex test-tube and cool in a bath of alcohol and solid $CO_2$ to $-35°$. Add in small portions, and with stirring, a solution of 7 cc. of phosphorus oxychloride in 30 cc. of cold ($-30$ to $-35°$) dry pyridine. Considerable heat is evolved but the temperature should be kept below $-20°$. After all the oxychloride is added, remove the mixture from the cooling bath and place in an ice-salt mixture at $-10$ to $-15°$ for 2 hours. During this time a sticky solid separates.

Remove from the cooling mixture, add about 40 gm. of chipped ice, and stir until dissolved. Combine four such lots, cool, and add cold 6 N sodium hydroxide solution with active stirring until alkaline to phenolphthalein. (About 220 cc. are required.) Two layers form. Decant the top layer and concentrate under reduced pressure to a small volume; add the lower layer and continue the

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[7] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **80**, 633 (1928)

[8] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **79**, 621 (1928).

evaporation until no odor of pyridine can be noticed. (It may be necessary to add water from time to time.)

Dilute to 500 cc. and add, with stirring, a solution of 80 gm. of $BaCl_2 \cdot 2H_2O$ in 500 cc. of water. Remove the precipitate by centrifugation, concentrate the solution to 500 cc. under reduced pressure, and suction filter using a small amount of charcoal.

Add with stirring an equal volume of 95 per cent alcohol. Allow to stand $\frac{1}{2}$ to 1 hour, filter with suction, and wash the precipitate with 50 per cent alcohol.

Redissolve the precipitate in water, concentrate to 500 cc., filter using charcoal, and reprecipitate with an equal volume of 95 per cent alcohol. Wash with 50 per cent and 95 per cent alcohol. Air-dry and powder. Yield, 45 to 50 gm.

### B. Phosphorylation of Fructose.

The procedure for the phosphorylation of fructose is identical with that for glucose. The sugar dissolves readily in the pyridine on slight warming. Less precipitate forms than with the glucose on adding the barium chloride solution but the yield is somewhat less, 30 to 40 gm., and the product is faintly colored even after three precipitations.

### C. Phosphoric Ester from Diacetone Glucose.

*1. Preparation of Diacetone Glucose.*—To 2 liters of acetone (u.s p.) containing 1 per cent of dry HCl gas add 100 gm. of dextrose[9] and shake in the machine for 48 hours. Filter off the unchanged sugar, immerse in running water, and pass in dry $NH_3$ gas till faintly alkaline to moist litmus. Filter using suction, and wash the cake with acetone. Concentrate the acetone solutions under reduced pressure to a very thick syrup and extract repeatedly with boiling ligroin (80–90°). Clarify with charcoal, filter hot, and allow to crystallize in the ice box. Filter off the crystals and air-dry. Yield, 45 to 50 gm.

*2. Phosphorylation of Diacetone Glucose.*—Dissolve 20 gm. of diacetone glucose in 100 cc. of dry pyridine and cool to −40 to −50°. Add, in one portion, a solution of 6.5 cc. of phosphorus oxychloride in 25 cc. of cold (−30 to −35°) dry pyridine. Re-

[9] A commercial product (Cerelose, Corn Products Company), mostly α-glucose, was used.

move the mixture from the cooling bath, stir for a few moments, and place in an ice-salt mixture at $-10$ to $-15°$ for 2 hours.

Recool to $-35°$ and add, in 2 cc. portions, a 10 per cent solution of water in pyridine, cooling between each addition and keeping the temperature below $-20°$. After 12 to 14 cc. have been added the heat evolution diminishes greatly. At this point add chipped ice with stirring and then 30 to 40 cc. of ice water.

Combine four such batches and add, slowly and with stirring, a cooled (40°) barium hydroxide solution (250 gm. of $Ba(OH)_2 \cdot 8H_2O$ in 300 cc. of boiling water) until the mixture is faintly alkaline to thymolphthalein. Only a slight precipitate forms. Add a small amount of decolorizing carbon and filter with suction.

Concentrate under reduced pressure to about 300 gm. total weight and adjust to pH 4.0 to 4.2 (brom-phenol blue) with 6 N HCl. Add 1 liter of acetone and shake vigorously. If any lumps remain, grind to a paste in a mortar, readjusting the pH if necessary. A total of about 45 cc. of 6 N HCl is required. Suction filter and wash the barium chloride precipitate thoroughly with 90 per cent acetone. Combine the filtrate and washings, concentrate under reduced pressure to a very thick syrup, and dissolve with shaking in 500 cc. of acetone. Add a small amount of charcoal and suction filter.

To the acetone solution add an equal volume of absolute ether, stir, and allow to settle. Decant off the ether-acetone and free the sticky solid of mother liquor by repeatedly stirring and decanting. Reextract the sticky solid with absolute ether to remove all traces of free diacetone glucose. Air-dry and powder. The product is slightly yellow. Yield, 70 to 80 gm.

If desired, the acid barium salt may be converted to the basic salt as follows: Dissolve the sticky solid, obtained above, in 500 cc. of water and concentrate under reduced pressure until the ether and acetone are removed. Add a warm saturated solution of barium hydroxide until faintly alkaline to thymolphthalein. Concentrate under reduced pressure to about 500 cc. and suction filter, using charcoal.

Add an equal volume of acetone and stir at intervals until a gel forms and becomes quite solid. Place in the ice box for 24 hours with occasional stirring to break up the mass. Suction filter and wash with 50 per cent water-acetone, followed by acetone, and

finally ether. Air-dry and powder. The product is perfectly white. Yield, 40 to 50 gm.

*3. Removal of Acetone Groups by Hydrolysis.*—Dissolve 50 gm. of the barium salt (basic) in 500 cc. of 1.0 N hydrochloric acid and keep at 38–40° for 48 hours. Add 6 N sodium hydroxide slowly and with stirring until faintly alkaline to phenolphthalein and then a solution of 5 gm. of barium chloride in a small amount of water. Concentrate under reduced pressure to 200 cc., and suction filter, using charcoal. Precipitate with an equal volume of 95 per cent alcohol, adding it slowly and with stirring. Suction filter and wash the precipitate with 50 per cent alcohol. Dissolve the solid in water and concentrate under reduced pressure to 200 cc. Filter and reprecipitate as above. Wash with 50 per cent and 95 per cent alcohol. Dry in air. The product is perfectly white. Yield, 40 gm.

### D. Phosphoric Ester from α-Diacetone Fructose.

*1. Preparation of α-Diacetone Fructose.*[10]—To 2 liters of acetone (U.S.P.) add 10 cc. of concentrated sulfuric acid. Mix well, add 100 gm. of powdered fructose,[11] and shake in the machine until practically all is dissolved (about 24 hours, but this depends upon the temperature). Without filtering, cool in running water and pass in dry ammonia gas until faintly alkaline to moist litmus. Filter with suction and concentrate under reduced pressure to a thick syrup.

Dissolve in 300 cc. of ether and wash the ether solution by shaking with three 100 cc. portions of 20 per cent sodium hydroxide and then one 25 cc. portion of 40 per cent sodium hydroxide. Dry with anhydrous sodium sulfate and filter. Concentrate the ethereal solution under reduced pressure to 175 cc. volume and cool in the ice box. Filter off the crystals, concentrate the filtrate to a syrup and recool. Again filter and combine the crystals with the first crop. (The residual material is a mixture of α- and β-diacetones and their recovery is not easy.) Yield, 55 gm. Recrystallize, using 500 cc. of ligroin (80–90°) for each 100

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[10] The general method employed is that of Ohle, H , and Koller, I , *Ber chem Ges* , **57**, 1566 (1924)

[11] "Levulose, Pure" Eimer and Amend, was found to be very satisfactory. It was used throughout this work without further purification.

gm. of crude dry material. The product often contains some of the $\beta$ isomer and should be recrystallized until pure ($[\alpha]_D^{20} = -160.7°$).

*2. Phosphorylation of α-Diacetone Fructose.*—Phosphorylate α-diacetone fructose exactly as for diacetone glucose, combine four lots, and use an identical procedure through the neutralization with barium hydroxide. After filtering, concentrate under reduced pressure to 500 cc. Make sure the solution is still faintly alkaline to thymolphthalein and suction filter after adding charcoal.

Add an equal volume of 95 per cent alcohol, stir several minutes until precipitation takes place, and allow to stand for one-half hour. Suction filter and wash with 50 per cent alcohol. Extract the washed precipitate with 2 to 3 liters of cold water in several portions and when all is dissolved, filter using charcoal and concentrate under reduced pressure to 700 cc. volume. Frothing may be prevented with a few drops of octyl alcohol. Add an equal volume of 95 per cent alcohol and stir as before. After standing 1 to 1½ hours, filter with suction and wash the precipitate thoroughly with 50 per cent alcohol, followed by 95 per cent alcohol, and finally ether. Dry and powder. Yield, 75 to 85 gm.

*3. Removal of Acetone Groups by Hydrolysis.*—Dissolve 50 gm. of barium α-diacetone fructose phosphate in 500 cc. of 0.5 N HCl and keep at 38–40° for 10 hours. Add the sodium hydroxide and barium chloride as for the diacetone glucose phosphate and centrifuge. Suspend the precipitate in water to which 2 to 3 cc. of saturated barium hydroxide solution have been added and again centrifuge. Combine the solutions and concentrate to 100 cc. volume under reduced pressure. Suction filter using a small amount of charcoal and precipitate as for the diacetone glucose but use 3 volumes of alcohol. Wash the precipitate with 70 per cent alcohol, dissolve in water, concentrate as before to 60 cc., and filter. Reprecipitate with 1 volume of 95 per cent alcohol. Wash and dry as for the diacetone glucose phosphate. Yield, 20 to 25 gm.

### E. Phosphoric Ester from β-Diacetone Fructose.

*1. Preparation of β-Diacetone Fructose.*[10]—To 2 liters of acetone (U.S.P.) slowly add 80 cc. of concentrated sulfuric acid and mix well. Add 100 gm. of powdered fructose and shake in the machine

for 3 to 5 hours until dissolved.   Without filtering, cool in running water and pass in ammonia gas until faintly alkaline to moist litmus.

Filter with suction, wash the cake with acetone, and concentrate the combined filtrate and washings under reduced pressure to a thick syrup.   Without allowing the mass to crystallize, dissolve with 100 cc. of chloroform.   Add 400 cc. of ligroin (80–90°) previously warmed to 35°.   Stir well, add a small amount of charcoal, and filter immediately using suction.

Allow the solution to stand overnight in the ice box and filter off the crystals.   Concentrate the solution under reduced pressure to about 150 cc. volume, allow to cool to 30–35°; a second crop of crystals is formed which is filtered off.   Cool the filtrate overnight in the ice box; a third crop of crystals is formed.   (The residual syrup contains more $\beta$-diacetone but the recovery is tedious.) Combine the three crops and air-dry.   Yield, 85 to 90 gm.

To 100 gm. of the crude material add 1 liter of boiling ligroin (80–90°), stir, and decant.   Reheat the solution almost to boiling and again extract and decant.   A dark-colored sticky material remains undissolved if the extraction is rapidly performed and the solvent is not too hot.

Heat the extract to boiling, add charcoal, and filter.   Cool to room temperature and allow to crystallize.   Yield, 80 gm. Several 100 gm. lots may be recrystallized from the same mother liquor which is then discarded.

*2. Phosphorylation of $\beta$-Diacetone Fructose.*—Dissolve 20 gm. of $\beta$-diacetone fructose in 100 cc. of dry pyridine and cool to $-45$ to $-50°$.   Add one-half of a solution of 6.5 cc. of phosphorus oxychloride in 25 cc. of cold ($-30$ to $-35°$) dry pyridine.   Stir well, recool to $-40°$, and add the remainder of the oxychloride solution. Stir for a few minutes and place in an ice-salt mixture at $-10$ to $-15°$ for 2 to $2\frac{1}{2}$ hours.

Recool to $-35°$ and treat as for diacetone glucose.   After combining four batches, make alkaline with barium hydroxide as before, and filter.   Concentrate under reduced pressure to 400 cc. or until frothing interferes.   In the same flask add 4 volumes of acetone and shake occasionally for 15 to 20 minutes.   Filter off the barium chloride with suction and wash with 80 per cent acetone. Concentrate the combined acetone solutions under reduced

pressure to a syrup and pour into a shallow crystallizing dish. Allow to air-dry with occasional stirring to break up the surface crust. Powder, dry in a vacuum desiccator over $CaCl_2$, repowder, and extract several times with acetone or ether. Redry. Yield, 100 to 120 gm.

*3. Removal of Acetone Groups by Hydrolysis.*—Use 50 gm. of barium $\beta$-diacetone fructose phosphate, 500 cc. of 1 N HCl, and keep at 38–40° for 24 hours. Thereafter employ the same procedure as for the $\alpha$-diacetone derivative but use only 1 volume of 95 per cent alcohol for the first precipitation and wash the cake with 50 per cent alcohol. The rest of the procedure is identical with that for the $\alpha$-diacetone derivative. Yield, 12 to 15 gm.

## II. *Hydrazone and Osazone Formation.*

The procedures described below for preparing the hydrazones and osazones were the most successful of several which were tried. In those cases in which no products were obtained by the method given, it was not found possible to secure them by any other technique which was tried.

A weighed amount of the pure barium salt of the ester was dissolved in water, cooled with ice, and the barium was quantitatively precipitated with sulfuric acid. The mixture was diluted to correspond to 12 gm. of dry barium salt per 100 cc. (0.3 molal) and was then centrifuged until clear.

For preparing the hydrazones, 1.8 cc. of pure phenylhydrazine (0.018 mols) were added to 20 cc. (0.006 mols) of the barium-free solution prepared as above. After standing a few minutes at room temperature the mixture was cooled and stirred. When crystallization started an equal volume of 95 per cent alcohol was added and the cooling and stirring were continued for several minutes. The mixture was then suction-filtered and the residue was well washed with 50 per cent alcohol followed by alcohol-ether (1:1) and finally ether. The product was immediately placed over phosphorus pentoxide in a highly evacuated desiccator and allowed to dry in the cold room.

For preparing the osazones, 2 cc. of glacial acetic acid, 25 cc. of water, and 3 cc. of pure phenylhydrazine (0.030 mols) were added to 25 cc. (0.0075 mols) of the barium-free solution prepared as previously described. The mixture was heated in a boiling

water bath and every few minutes was removed and cooled. Any osazone which had formed was filtered with suction and the heating was continued until practically no more osazone formed, a total time of about 1 hour. The collected osazone was dried and weighed and then recrystallized as indicated.

The results with the various esters were as follows:

### A. Phosphoric Ester from Phosphorylation of Glucose.

*1. Hydrazone.*—No insoluble hydrazone.

*2. Osazone.*—Only a small amount of dark tarry material was obtained and no crystalline product was isolated from the tar.

### B. Phosphoric Ester from Diacetone Glucose.

*1. Hydrazone.*—No insoluble hydrazone.

*2. Osazone.*—The crude osazone obtained after cooling overnight in the ice box was a dark tarry material. It was once recrystallized from aqueous acetic acid and twice from aqueous isopropyl alcohol. The analysis corresponded to an anhydrohexosazone.

5.080 mg. substance: 11.795 mg. $CO_2$ and 2.770 mg. $H_2O$.
3.850 "      "     :   0.572 cc. $N_2$ (754 mm. and 30°).
$C_{18}H_{20}O_3N_4$.   Calculated.   C 63.47, H 5.92, N 16.47.
               Found.     " 63.31, " 6.10, " 16.59, P none.

The product when slowly crystallized formed clusters of slender canary-yellow needles. Like the osazone of Levene and Sobotka[4] it was very soluble in methyl alcohol. The rotation was close to that previously found.[5]

$$[\alpha]_D^{28} = \frac{-0.73° \times 100}{0.5 \times 1.0} = -146°.$$

Due apparently to traces of the tarry impurity, the substance had a tendency to darken in the air and the melting point was 156–159° which is a little lower than that previously reported.

### C. Phosphoric Ester from Phosphorylation of Fructose.

*1. Hydrazone.*—No insoluble hydrazone.

*2. Osazone.*—The yield of crude dark material was 1.1 gm. (40 per cent theoretical). After twice recrystallizing from aque-

ous pyridine, the product was extracted with hot methyl alcohol in which it was only slightly soluble. The final product was a felt of curved canary-yellow needles. The analysis corresponded to a hexosazone.

4.595 mg. substance: 10.220 mg. $CO_2$ and 2.530 mg. $H_2O$.
4 870  "      "     :  0.671 cc. $N_2$ (749 mm. and 23°).
$C_{18}H_{22}O_4N_4$.  Calculated.  C 60.31, H 6.19, N 15.64.
                      Found.      " 60.65, " 6.16, " 15.59, P none.

The substance melted at 203–204° with decomposition as did an intimate mixture with a sample of pure glucosazone.

### D. Phosphoric Ester from α-Diacetone Fructose.

*1. Hydrazone.*—The yield was 1.2 gm. (40 per cent theoretical) and the product was practically colorless. The analysis corresponded to a diphenylhydrazine salt of a phosphohydrazone.

4.290 mg. substance:  8.010 mg. $CO_2$ and 2 440 mg. $H_2O$.
5 455  "      "     :  0.670 cc. $N_2$ (766 mm. and 28°).
5 595  "      "     : 20.930 mg. ammonium phosphomolybdate (Pregl).
$C_{24}H_{35}O_8N_5P$.  Calculated.  C 50.86, H 6.23, N 14.84, P 5.48.
                      Found.      " 50.89, " 6.29, " 14.16, " 5.46.

The product melted at 96–98° and decomposed at 123–125° with effervescence. Its optical rotation in pyridine-methyl alcohol (1:1) 5 minutes after solution was −50.6° and at equilibrium (after 24 hours in the cold room) was

$$[\alpha]_D^{25} = \frac{-1.75° \times 100}{2 \times 2.50} = -35.0°.$$

*2. Osazone.*—The osazone of this ester has been previously described[5] but the results may be repeated here for completeness. The yield of crude product was 1.3 gm. (48 per cent theoretical). After two recrystallizations from aqueous pyridine and one from methyl alcohol, it formed a felt of curved canary-yellow needles. The analysis corresponded to an anhydrohexosazone.

4.940 mg. substance: 11.495 mg. $CO_2$ and 2.590 mg. $H_2O$.
5 351  "      "     :  0.759 cc. $N_2$ (762 mm. and 22°).
$C_{18}H_{20}O_3N_4$.  Calculated.  C 63.47, H 5.92, N 16.47.
                      Found.      " 63.45, " 5.86, " 16.44, P none.

It contracted at 160° and melted and darkened at 165–168° without effervescence. In pyridine-methyl alcohol (1:1) 5 minutes after solution its rotation was

$$[\alpha]_D^{20} = \frac{-0.55° \times 100}{0.5 \times 0.8} = -138°.$$

*E. Phosphoric Ester from β-Diacetone Fructose.*

*1. Hydrazone.*—The yield was 1.0 gm. (33 per cent theoretical) and the product was practically colorless. The analysis indicated a phenylhydrazine salt of a phosphohydrazone.

0 0722 gm. substance: 7.60 cc. $N_2$ (760 mm. and 28°).
0 2000 " " : 0.0480 gm. $Mg_2P_2O_7$.
$C_{18}H_{27}O_8N_4P$. Calculated. N 12 23, P 6 77.
Found. " 12 34, " 6 68.

The product started to brown at 93–95°, sintered at 96–97°, contracted and blackened at 107–110°. There was no effervescence up to 180°. Its optical rotation in pyridine-methyl alcohol (1:1) 5 minutes after solution was −15.0° and at equilibrium (after 50 hours in the cold room) was

$$[\alpha]_D^{25} = \frac{-1.68° \times 100}{2 \times 2.50} = -33.6°.$$

*2. Osazone.*—This osazone formed with the greatest ease. Yield, 2.0 gm. (74 per cent theoretical). After two recrystallizations from aqueous pyridine, the material formed a felt of curved lemon-yellow needles. The analysis corresponded to a hexosazone.

0.0666 gm. substance: 9.20 cc. $N_2$ (761 mm. and 23°).
$C_{18}H_{22}O_4N_4$. Calculated. N 15.63. Found. N 15.95, P none.

It melted and decomposed at 203–204° as did an intimate mixture with a sample of pure glucosazone.

# BASIC AMINO ACIDS.

## THE ESTIMATION OF THE BASIC AMINO ACIDS IN SMALL AMOUNTS OF CASEIN AND EDESTIN BY THE MODIFIED METHOD OF VICKERY AND LEAVENWORTH AND OTHER METHODS.*

By H. O. CALVERY.

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Recently Vickery and Leavenworth (1) published a modified method for the estimation of the basic amino acids of edestin which is without doubt the most accurate yet described. These authors (2) have since used the method, with slight modifications, for the estimation of the bases of hemoglobin. The later modifications were necessary because of the relatively large amount of histidine and small amount of arginine present in hemoglobin. Vickery and Leavenworth have recommended that at least 50 gm. of protein be used for analysis. However, such large quantities of protein are not always easily available. This was particularly true in the case of the protein material available from the developing hen's egg which the author wished to investigate by this method. After some study of their method, there appeared no reason why, if carefully executed, it could not be applied to smaller quantities of protein with a considerable degree of accuracy. The present investigation was undertaken to determine the accuracy of the method when applied to small amounts of casein and edestin. It was advisable to make such an investigation on well known proteins, including one which had already been studied, with larger quantities, by the same method.

Since several changes in the procedure of Vickery and Leavenworth have been made, and since the detailed method has not

been published, it seemed advisable to present the complete details of the method used in this investigation. The method has been supplemented, for comparison, by the Hanke and Koessler (3) colorimetric method as modified by Hanke (4) and the Plimmer and Phillips (5) bromination method for histidine and by the alkali hydrolysis method of Van Slyke (6) as modified by Plimmer (7) and by Koehler (8) for arginine.

### EXPERIMENTAL.

### *Method.*[1]

1. Hydrolyze 5 gm. of the material (it is best to make duplicate determinations) being investigated with 50 cc. of 20 per cent hydrochloric acid for 36 hours over a free flame. Transfer the hydrolysate quantitatively to a distilling flask, add 1.5 cc. of concentrated sulfuric acid,[2] and remove most of the hydrochloric acid by concentration to a thin syrup under reduced pressure on a steam bath. Take the syrup up in water and transfer it to a 50 cc. volumetric flask and determine the total nitrogen in an aliquot by the Kjeldahl-Gunning method.[3]

2. Transfer the remainder of the solution to a 250 cc. centrifuge bottle and add cold saturated barium hydroxide until the solution is distinctly alkaline to litmus.[4] Centrifuge to remove the

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[1] The papers of Vickery and Leavenworth should be consulted for more detailed theoretical discussions of several points.

[2] The addition of sulfuric acid may be omitted, if desired; however, its addition greatly reduces the amount of silver oxide required since more hydrochloric acid is removed in the distillation. Care must be taken not to evaporate to a thick syrup since losses may occur due to slight charring by the sulfuric acid. If the evaporation is repeated, nearly all the hydrochloric acid is removed.

[3] Some proteins yield large quantities of acid-insoluble melanin. After determination of the total nitrogen on the protein hydrolysate, the acid melanin should be filtered off, washed thoroughly, and discarded, while the washings should be added to the filtrate.

[4] If alkali-insoluble melanin is not removed, the greater part of it is apparently carried down with the precipitate of silver chloride as is indicated by a removal of most of the color from the solution. However, if the acid concentration is too high, the melanin is not precipitated along with the silver chloride and interferes somewhat with the Hanke and Koessler method, but more so with the bromination method.

*alkali-insoluble melanin,* wash the precipitate twice with small amounts of very dilute barium hydroxide, and add sulfuric acid to the combined filtrate and washings until a *strong acid reaction to Congo red paper* is obtained. Keep the solution distinctly acid to Congo red with sulfuric acid and add silver oxide in small amounts with constant stirring, until all the chlorides have been precipitated. (If purines are present they will be largely precipitated at this point.) Centrifuge to remove the silver chloride and wash twice with hot water. Concentrate the filtrate and washings on a steam bath to 200 cc. and remove the arginine and histidine by the following procedure.

3. Keep the solution *distinctly acid* as before and add silver oxide until a drop of the solution added to a barium hydroxide solution on a watch-glass gives a brown precipitate. (Considerable silver oxide is usually required at this point.) When the required amount of silver oxide is present, add hot saturated barium hydroxide until the solution is strongly alkaline to phenolphthalein paper. Stir for 15 minutes, centrifuge to remove the precipitate, and wash once with water made alkaline with barium hydroxide. The precipitate contains the arginine and histidine with small amounts of other amino acids which are precipitated as silver salts. Acidify the filtrate and washings and set aside as the *crude lysine fraction.*

4. Suspend the silver salts of arginine and histidine in hot water, make the suspension acid with sulfuric acid, and precipitate the silver as silver sulfide. (Stir thoroughly and test for excess hydrogen sulfide since losses often occur at this point.) Centrifuge to remove the silver sulfide, wash the precipitate thoroughly with hot water, and, if necessary, concentrate the solution and washings on a steam bath to 200 cc. If the hydrogen sulfide is not removed by the procedure, the solution should be aerated for its complete removal.

5. Again add silver oxide in excess, *the acidity being maintained as before.* Add cold saturated barium hydroxide slowly with stirring, until the solution has been brought to pH 7.4[5] as indicated

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[5] Rosedale (9) in a recent investigation of the amino acids of flesh has advocated the precipitation of histidine as the silver salt in 1.5 per cent sulfuric acid. In a comparison of the values obtained by his method with those obtained by the Vickery and Leavenworth (1) procedure, Rosedale

by brom-thymol blue. At this point the precipitate settles
sharply. The acidity may be roughly followed by allowing a drop
of the indicator occasionally to fall on the surface of the liquid.
At this pH the histidine silver is very insoluble and precipitates,
while most of the arginine remains in solution. As Vickery and
Leavenworth (1) have pointed out, solution and reprecipitation
are the most effective way of getting the histidine fraction free of
arginine with very little loss of histidine because of the insolubility
of its silver salt. Therefore, after 15 minutes centrifuge and,
without washing, suspend the histidine silver in water and remove
the silver by the addition of 10 per cent hydrochloric acid until
no more silver chloride is formed, boiling vigorously to insure
complete decomposition. The chlorides need not be removed at
this point. Cool the solution and add silver oxide and sulfuric
acid in excess as before. Add cold saturated barium hydroxide
until the solution has again been brought to pH 7.4, stir for 15
minutes, and centrifuge. Save the filtrate and add it to the filtrate
from the precipitate formerly obtained at pH 7.4. Suspend the
precipitate in hot water, add an excess of 10 per cent hydrochloric
acid (a test for an excess of hydrochloric acid can best be made
by addition of a drop of a solution of silver sulfate), and boil
vigorously. Centrifuge to remove the chlorides, wash thoroughly
with hot water, and concentrate the filtrate and washings to
100 cc. This is the *crude histidine fraction*. The filtrates from
the precipitations made at pH 7.4 contain the arginine.

6. Concentrate a 50 cc. aliquot of the crude histidine fraction
to a syrup *in vacuo* to remove the excess hydrochloric acid. Pre-
cipitate the remaining chlorides with a very small excess of
sulfuric acid and silver oxide. Centrifuge to remove the silver
chloride and wash thoroughly with hot water. Remove the
excess silver with hydrogen sulfide, centrifuge, wash with hot
water, and aerate the combined filtrate and washings to remove the
excess hydrogen sulfide. Concentrate the solution to such an
extent that it contains 0.5 to 1 per cent of histidine (usually to
about 25 cc). The volume desired may be approximately de-

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(10) finds that his method gives much lower values for histidine and higher
values for arginine. It is possible that all the histidine was not pre-
cipitated in the 1.5 per cent sulfuric acid used by Rosedale.

termined by calculation of the total nitrogen of the crude histidine fraction. Add enough sulfuric acid so that the solution, after addition of two-thirds its volume of Hopkins' reagent, has an acid concentration which does not exceed 5 per cent by weight of sulfuric acid[6] (see "Discussion").

After 48 hours centrifuge and wash the precipitate twice with small amounts of Hopkins' reagent diluted with 3 volumes of water. Discard the filtrate and washings. Remove the mercury from the precipitate with hydrogen sulfide, centrifuge, and wash with hot water. Boil to remove the excess hydrogen sulfide from the combined filtrate and washings and concentrate to 50 cc. This is the *purified histidine fraction*.

7. Take two 5 cc. aliquots for total nitrogen determination by the Kjeldahl-Gunning method and calculate the amount of flavianic acid, 2,4-dinitro-1-naphthol-7-sulfuric acid, required to precipitate the histidine (1 gm. of histidine nitrogen requires 14.96 gm. of reagent). Concentrate the remainder of the solution to approximately 5 cc. and add to the boiling solution a very small excess of a 5 per cent solution of flavianic acid in water. Filter off, on a weighed crucible, the first precipitate which forms on standing overnight. Concentrate the filtrate to one-fifth the original volume and a second crop of crystals will usually appear on standing which should be added to the first precipitate and weighed. ᐧ The melting point of the pure salt according to Vickery (11) is 251–252°, and, if prepared exactly according to directions, the composition is $C_6H_9N_3O_2 \cdot 2C_{10}H_6N_2SO_3$ when dried at 105°. Multiply the weight of the salt obtained by 0.1979 to get the equivalent of histidine in the sample used and correct for aliquots removed to get the total histidine in the original material.

8. Use two 10 cc. aliquots of the crude histidine fraction for total nitrogen determination by the Kjeldahl-Gunning method. Use two 5 cc. aliquots for the estimation of histidine by the bromination method of Plimmer and Phillips (5). Use 1 cc. aliquots and dilute according to requirements for the estimation of histidine by the method of Hanke and Koessler (3) as modified by Hanke

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[6] According to Hopkins' original directions the reagent consists of 10 gm. of mercuric sulfate, 5 cc. of concentrated sulfuric acid, and enough water to make a total volume of 100 cc. The solubility of the mercury salt of histidine varies with the acid concentration as is shown in Table IV.

(4). This method may also be applied to the purified histidine fraction since it requires such small amounts of material. It was used in this investigation in the case of casein.

9. Combine the filtrates from the two silver precipitations at pH 7.4, make acid to Congo red paper, and concentrate to 200 cc. An excess of silver should be present in the solution and, if not, it should be added as before   Add warm saturated barium hydroxide until the solution is strongly alkaline to phenolphthalein paper, centrifuge, and wash the precipitate once with water made alkaline with barium hydroxide.   Acidify the filtrate and washings with sulfuric acid and add to the *crude lysine fraction.*   Suspend the precipitate in water and acidify with sulfuric acid to the faintest possible reaction to Congo red paper,[7] remove the silver as silver sulfide, and wash with hot water.   Concentrate the filtrate and washings to 50 cc. and test for complete removal of hydrogen sulfide.   Estimate the total nitrogen in two 5 cc. aliquots by the Kjeldahl-Gunning method.   Use two 5 cc. aliquots for estimation of arginine by the method of Van Slyke (6), with the modified apparatus of Koehler (8) and the modified procedure of Plimmer (7).

Treat a 25 cc. aliquot, at the boiling point, with a small excess of a 5 per cent solution of flavianic acid.   The amount of flavianic acid required can be calculated from the total nitrogen of the arginine fraction, since 1 gm. of arginine nitrogen requires 5.61 gm. of the reagent.   Allow to crystallize in two successive crops as specified for histidine above.   The weight of the arginine flavianate, dried at 100° and multiplied by 0.3566, gives the equivalent of arginine in the solution used, from which the amount present in the original sample can be calculated when corrections are made for all aliquots removed.

10. The combined crude lysine fractions contain the ammonia and practically all the amino acids except the histidine and arginine and should be acid to Congo red paper.   Remove the

[7] When the silver salt of arginine is decomposed with hydrogen sulfide, it is better to add an excess of sulfuric acid in order to obtain a more rapid and complete decomposition.   Then after the removal of the hydrogen sulfide by aeration or boiling, the excess sulfuric acid is removed with barium carbonate.   The excess sulfuric acid must be removed since it prevents the quantitative precipitation of arginine flavianate.

silver as the sulfide and wash thoroughly. Concentrate the filtrate and washings to 200 cc., make alkaline with barium hydroxide, centrifuge, and wash the barium sulfate several times with hot water. Add 100 cc. of alcohol to the combined filtrate and washings and concentrate on a steam bath under reduced pressure to 150 cc. to remove the ammonia. Make the solution distinctly acid with sulfuric acid and add barium carbonate to remove the excess of sulfuric acid,[8] centrifuge, and wash the precipitate twice. Concentrate the filtrate and washings to 75 cc., add enough concentrated sulfuric acid (2.8 cc., sp. gr. 1.86) to make the solution 7 per cent, and then add 8 gm. of phosphotungstic acid in 10 cc. of hot water. Let stand overnight, centrifuge, and wash the precipitate twice with small amounts (30 cc.) of 2 per cent phosphotungstic acid in 5 per cent sulfuric acid. Dissolve the precipitate in 60 cc. of acetone and dilute to 150 cc. with water. Make the solution strongly alkaline with barium hydroxide, centrifuge, and wash the precipitate of barium phosphotungstate several times with dilute barium hydroxide. The filtrate and washings should be strongly alkaline to phenolphthalein paper and clear. Acidify strongly with sulfuric acid and add barium carbonate to remove the excess sulfuric acid,[8] centrifuge, and wash thoroughly. Concentrate the solution and washings to 50 cc. This is the *purified lysine fraction.*

Determine the nitrogen in two 5 cc. aliquots by the Kjeldahl-Gunning method. Concentrate the remainder of the solution on a steam bath to 5 cc., add absolute alcohol to faint turbidity, then add about two-thirds of the calculated amount of a saturated solution of picric acid in absolute alcohol required to form the lysine monopicrate, and allow to crystallize overnight. Filter,

[8] Barium carbonate has proved a very useful reagent in this procedure, since on several occasions it is necessary to remove completely both the sulfuric acid and the barium. The use of barium carbonate enables one to remove effectively the excess sulfuric acid without forming barium salts with the amino acids. It was found on several occasions that after addition of sulfuric acid, until the solution was distinctly acid to litmus, barium was still present in the solution and could be removed by the addition of more sulfuric acid. This difficulty was not encountered if the solution was made strongly acid with sulfuric acid, and the excess removed with barium carbonate. The barium carbonate should be free of barium hydroxide which, if present, can be removed by washing.

and to the filtrate add one-half of the remainder of the calculated amount of the picric acid solution. If a precipitate forms, repeat the process.[9] Wash the lysine picrate with alcohol and ether, dry and weigh. If impure, it should be recrystallized from a minimal amount of water and corrected for solubility (0.54 gm. per 100 cc. of water). A satisfactory picrate should decompose with slight explosion above 250° (12). The weight of the picrate obtained multiplied by 0.3855 gives the amount of free lysine.

The mother liquors in the various procedures described above have not been examined as Vickery and Leavenworth have recommended when using large quantities of protein, therefore the values reported are probably not the maximum values that can be obtained. However, they are of sufficient accuracy to indicate that the method may be of great value when small quantities only of protein are available. The data obtained by this method and the others used in this investigation are recorded in Tables I and II. Samples of the arginine flavianate and histidine flavianate as prepared by the above procedure from casein were analyzed for nitrogen.

*Arginine Flavianate.*

Micro-Dumas-Pregl for nitrogen (13).
4.079 mg. gave 0.633 cc. N at 24° and 743 mm.
Required for $C_6H_{14}N_4O_2 \cdot C_{10}H_6N_2SO_8$.    N 17.21.
Found.                                          "  17.26.

*Histidine Flavianate.*

Micro-Dumas-Pregl for nitrogen (13).
2.296 mg. gave 0.254 cc. N at 23° and 743 mm.
Required for $C_6H_9N_3O_2 \cdot 2C_{10}H_6N_2SO_8$.    N 12.52.
Found.                                          "  12.49.

[9] In the precipitation of lysine as the monopicrate the procedure recommended is best for three reasons: first, the solution often contains appreciable quantities of other amino acids precipitated by phosphotungstic acid, thus other nitrogen than lysine nitrogen is present; second, if an excess of picric acid is added, lysine dipicrate is formed which is very soluble in both alcohol and water, thus a semicrystalline mass is usually formed and the lysine cannot be quantitatively recovered; third, when a large excess of sulfuric acid is present in the solution, the lysine picrate does not quantitatively precipitate.

## DISCUSSION.

Casein was selected for this investigation, because it is a common protein which has been investigated many times by various methods for the basic amino acid content.    Edestin was selected

TABLE I.

*Arginine and Lysine Content of Casein and Edestin.*

The values are expressed in per cent of the total nitrogen present in the solution after hydrolysis.    The proteins were not analyzed before hydrolysis.

| Experiment No | Sample. | Arginine N. | | | Lysine N. | |
| | | Total N in arginine fraction. | Alkaline hydrolysis method, Van Slyke. | Isolation as flavianate. | Total N in lysine fraction. | Isolation as picrate. |
| --- | --- | --- | --- | --- | --- | --- |
| Casein. | | | | | | |
| | | *gm.* | | | | |
| 1 | 5 | 7.98 | | 6 10 | | 2.27 |
| 2 | 5 | 9.31 | 6.62 | 5.83 | | 3.99 |
| 3 | 5 | 9 34 | 6 66 | 6.13 | 10.36 | 6.42 |
| 4 | 5 | 10.10 | 6 56 | 6.10 | 10.48 | 6.77 |
| 5 | 50 | 9 34 | 6 79 | 6.21 | 10.94 | 6 49 |
| 6 | 2.5 | 9 90 | 6.06 | 5.88 | 10.13 | 6.07 |
| 7 | 5 | 8 24 | 6 78 | 6.47 | 10.44 | 6.46 |
| 8 | 5 | 7.12 | 6.78 | 6.26 | 10.39 | 6.56 |
| 9 | 5 | 8.09 | 6.46 | 4.62* | 10.52 | 6.59 |
| 10 | 5 | 8.40 | 6 46 | 6 31 | 10.34 | 6.49 |
| Edestin. | | | | | | |
| 1 | 5 | 28.31 | 26.24 | 25 52 | 4.98 | 3.46 |
| 2 | 5 | 29 29 | 26 11 | 25 68 | 5.12 | 3.58 |
| Vickery and Leavenworth. | 366 3 | | | 27 2 | | 3 80 |

* Part was lost in this run.

because it was the protein used by Vickery and Leavenworth (1) for their original investigation.    The selection of edestin was advisable since the values obtained by these authors could be used for comparison with the values obtained by the technique described above.    In order to test the method thoroughly many

experiments were performed of which only ten on casein and two on edestin are being reported in this paper. Of these, one was a 50 gm. sample and one was a 2.5 gm. sample of casein for comparison. The data reported in Tables I and II show that with 5 gm. samples practically the same values were obtained as with a 50

TABLE II.

*Histidine Content of Casein and Edestin.*

The values are expressed in per cent of total N.

| Experiment No. | Sample. | Total N histidine fraction. | Bromination, Plimmer and Phillips. | Hanke and Koessler method on original. | N precipitated by mercuric sulfate. | Hanke and Koessler method on mercury precipitate. | Isolation as flavianate. |
|---|---|---|---|---|---|---|---|
| | | | | Casein. | | | |
| | *gm.* | | | | | | |
| 1 | 5 | 6.02 | | 5.93* | 3.39 | | 2.83 |
| 2 | 5 | 6.24 | 5.90 | 6.50* | 3.48 | 3.01* | 2.92 |
| 3 | 5 | 6.23 | 5.86 | 6.50* | | 2.90* | |
| 4 | 5 | 6.72 | 5.92 | 6.22* | | 2.83* | |
| 5 | 50 | 6.27 | 6.13 | 6.19 | 3.43 | 3.51 | 3.01 |
| 6 | 2.5 | 6.11 | 5.91 | 6.38 | 2.96 | 3.48 | |
| 7 | 5 | 6.47 | 6.00 | 6.33 | 2.93 | 3.25 | 2.39 |
| 8 | 5 | 7.28 | 6.00 | 6.59 | 3.46 | 3.42 | 2.92 |
| 9 | 5 | 6.29 | 5.87 | 6.24 | 3.43 | 3.62 | 2.98 |
| 10 | 5 | 6.37 | 5.86 | 6.16 | 3.43 | 3.51 | |
| | | | | Edestin. | | | |
| 1 | 5 | 7.32 | | | 5.13 | | 4.98 |
| 2 | 5 | 7.67 | | | 5.18 | | 5.12 |
| Vickery and Leavenworth. | 366.3 | | | | | | 5.15 |

* These values were obtained from another sample.

gm. sample, while the 2.5 gm. sample gave slightly lower results. A smaller sample than 5 gm. is not recommended although it may be used if the experiment is carefully performed.

In most of the modifications of the original Kossel and Kutcher (14) method for the estimation of the basic amino acids, three fractions are obtained; namely, the arginine fraction, the histidine

fraction, and the lysine fraction. In many of these modifications it has been considered that the only nitrogen present is that of the respective hexone base by which the fraction is designated. With this idea in mind many investigators have reported the total nitrogen of the histidine fraction, for example, as histidine. That this is not the case has been clearly shown by the work of Vickery and Leavenworth (1) who have isolated both glutamic and aspartic acids from this fraction while working with edestin. In the present investigation the total nitrogen of the fractions was estimated and the arginine and histidine were determined by more than one method on the respective fractions. In some cases the values obtained by different methods agree quite well, while in others they do not.

*Total Nitrogen of the Arginine Fraction.*—The total nitrogen of the arginine fraction obtained by the method described in this paper was higher than any values that have ever been reported for arginine in both casein and edestin. The total nitrogen of this fraction was quite variable, as shown in Table I, and it is probably obvious that it will vary with the pH at which the histidine is precipitated. In Experiment 8 on casein the histidine was precipitated at pH 7.4, while in all others it was precipitated at pH 7.0. The total nitrogen of the arginine fraction in this experiment was much lower than in any of the other experiments, while the total nitrogen of the corresponding histidine fraction was higher. It is evident from the values obtained by other methods that the total nitrogen of this fraction cannot be considered as arginine nitrogen.

*Alkaline Hydrolysis Method of Van Slyke for Arginine.*—The accuracy of this method for pure arginine can scarcely be questioned. The Plimmer (7) modification of the method and the Koehler (8) modification of the apparatus make it easy and accurate to use, especially when arginine alone is present in the solution or in the presence of small amounts of other amino acids. The values obtained by alkaline hydrolysis and reported in Table I are much smaller than the total nitrogen values, but they are very consistent and undoubtedly represent the maximum amount of arginine that is present in this solution. They are slightly higher than those obtained by isolation, which is what would be expected since an isolation method can never be absolutely quantitative.

*Isolation of Arginine as the Flavianate.*—Of all the compounds of arginine known at the present time arginine monoflavianate is the most insoluble in water.   It is one-half as soluble as arginine silver and only one-fourth as soluble as arginine phosphotungstate (15). It was first used in 1924 by Kossel and Gross (16) for the quantitative estimation of arginine, later by Kossel and Staudt (17) and by Vickery and Leavenworth (1).   The values recorded in Table I show how closely the isolation values correspond to those obtained by alkaline hydrolysis.   The analysis for nitrogen recorded also shows that the compound was quite pure and not contaminated with histidine flavianate.   Kossel and Staudt showed that it was not necessary to precipitate the arginine first with silver, but that the separation could be made directly.   This procedure would probably not be applicable in all cases, especially if histidine was present in large amounts.   It should be kept in mind that Kossel and Staudt were working with proteins in which the arginine was present in much larger quantities than histidine and that they subtracted the arginine nitrogen from the total nitrogen precipitated by silver and called the remainder histidine nitrogen. Vickery and Leavenworth (1, 2) preferred to make the separation by removal of the histidine as the silver compound at pH 7 or 7.4 and the subsequent precipitation of the arginine as the flavianate.

Although in most of the experiments reported in this paper the separation of arginine and histidine was made at pH 7, several experiments were made in which the separation was made at pH 7.4.   The latter is probably preferable in all cases and was recommended in the procedure described.   It has been used exclusively in an investigation of the basic amino acids of the chick embryo which will be reported in another paper.

The values obtained for arginine by alkaline hydrolysis and by isolation probably represent very nearly the actual amount of arginine present in casein, although Vickery and Leavenworth (1) showed that small amounts of arginine were obtained from the lysine fraction.

*Total Nitrogen of the Histidine Fraction.*—It is evident from the work of Vickery and Leavenworth (1) that this is not all histidine nitrogen, for they have been able to isolate other amino acids, notably aspartic and glutamic acids, from this fraction.   In their

work on edestin they found that less than half of the nitrogen of this fraction could be accounted for by the amount of histidine isolated.

*Method of Hanke and Koessler and Bromination Method of Plimmer and Phillips.*—The colorimetric method of Hanke and Koessler (3) is based on the fact that a colored complex is formed when histidine is treated with the diazo compound of sulfanilic

TABLE III.

*Bromination of Pure Histidine with Variations in Time, Temperature, and Acid Concentration.*

The volume was kept constant and titrations were made in 250 cc. volumetric flasks.

| Experiment No. | Sample | 20 per cent potassium bromide | Concentrated HCl. | 0.2 N NaBrO₃. | Temperature. | Time. | 10 per cent potassium iodide. | 0 1 N thiosulfate for back titration. |
|---|---|---|---|---|---|---|---|---|
| | $cc$ | cc. | cc. | cc. | °C. | min. | cc. | cc. |
| 1 | 5 | 10 | 10 | 5 | 20 | 10 | 10 | 8.40 |
| 2 | 5 | 10 | 10 | 5 | 20 | 15 | 10 | 8.40 |
| 3 | 5 | 10 | 10 | 5 | 20 | 20 | 10 | 8.30 |
| 4 | 5 | 10 | 10 | 5 | 20 | 25 | 10 | 8 30 |
| 5 | 5 | 10 | 10 | 5 | 20 | 60 | 10 | 8.10 |
| 6 | 5 | 10 | 2 5 | 5 | 20 | 15 | 10 | 8.15 |
| 7 | 5 | 10 | 5 | 5 | 20 | 15 | 10 | 8.13 |
| 8 | 5 | 10 | 10 | 5 | 20 | 15 | 10 | 8.40 |
| 9 | 5 | 10 | 20 | 5 | 20 | 15 | 10 | 8.35 |
| 10 | 5 | 10 | 30 | 5 | 20 | 15 | 10 | 8.40 |
| 11 | 5 | 10 | 30 | 5 | 20 | 15 | 10 | 8.45 |
| 12 | 5 | 10 | 10 | 5 | 5 | 15 | 10 | 9.22 |
| 13 | 5 | 10 | 10 | 5 | 5 | 15 | 10 | 9.35 |
| 14 | 5 | 10 | 10 | 5 | 20 | 15 | 10 | 8.40 |
| 15 | 5 | 10 | 10 | 5 | 50 | 15 | 10 | 5.95 |
| 16 | 5 | 10 | 10 | 5 | 50 | 15 | 10 | 6.00 |

acid in alkaline solution. The bromination method of Plimmer and Phillips (5) depends on the quantitative addition of 2 atoms of bromine to histidine in the presence of sodium bromate, potassium bromide, and hydrochloric acid. After 15 minutes potassium iodide is added and the iodine liberated by the excess bromine is titrated with standard thiosulfate. It is evident that both methods are subject to interfering substances, among which is

tyrosine. It is very interesting, if not significant, that in the work on casein, these two methods checked very consistently with the total nitrogen of that fraction. The fact that both methods sometimes yielded higher results than the total nitrogen was an indication that interfering substances were present. The bromination method is probably subject to the widest error since not only tyrosine but cystine and breakdown products of tryptophane interfere (5). Table III shows that temperature had a very marked influence on the absorption of bromine by histidine when a

TABLE IV.

*Standard Solutions of Pure Histidine in Sulfuric Acid Treated with an Excess of Hopkins' Reagent, and Various Dilutions Made.*

The concentration of sulfuric acid was estimated accurately by titration. The amount of material recovered was estimated as nitrogen and expressed in terms of total nitrogen present in the original solution. The experiments were continued for 48 hours.

| Experiment No. | Histidine solution. | Hopkins' reagent. | Water added. | Concentration of sulfuric acid by weight. | N recovered. | Nitrogen recovered after second dilution. |
|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | *cc.* | *per cent* | *per cent of total N* | *per cent* |
| 1 | 10 | 10 | None. | 17.12 | None. | 92.31 |
| 2 | 20 | 15 | 10 | 13.36 | " | 91.38 |
| 3 | 10 | 7 | 10 | 13.08 | " | 93.01 |
| 4 | 25 | 25 | None. | 10.17 | 9.55 | 92.82 |
| 5 | 25 | 25 | 5 | 9.60 | 32.26 | 93.91 |
| 6 | 25 | 25 | 15 | 8.30 | 45.23 | 92.87 |
| 7 | 10 | 10 | 18 | 5.72 | 91.33 | 93.76 |
| 8 | 10 | 10 | 25 | 4.20 | 93.80 | 94.22 |
| 9 | 10 | 10 | 25 | 4.26 | 93.60 | 94.03 |

solution of pure histidine dichloride was used, while the acid concentration and time of bromination also have some influence. The actual amount of histidine was indicated in Experiments 1, 2, 8, 10, and 14. In all experiments the volume before the addition of potassium iodide was 155 cc.

*Precipitation of Histidine with Mercuric Sulfate.*—Kossel and Patten (18) were the first to use this reagent for the quantitative separation of arginine and histidine. Osborne, Leavenworth, and Brautlecht (19) apparently did not believe that this method was

quantitative for they reversed the Kossel and Patten procedure. They precipitated first with mercuric sulfate, then precipitated the histidine left in solution as the silver compound. The results of Vickery and Leavenworth (1) on edestin were quite surprising, but they did not believe that more than half of the histidine had escaped precipitation with the mercuric sulfate. Their results on edestin were confirmed by the author and results, just as surprising, were obtained on casein. There is one very significant fact that must be kept in mind when histidine is being precipitated with Hopkins' reagent. *The acid concentration has a very marked influence on the solubility of the mercury salt of histidine.* This was demonstrated by the results reported in Table IV.

When this investigation was first begun very inconsistent and often very low results were obtained for histidine. The loss occurred during the precipitation with mercuric sulfate and was apparently due to the acid concentration. It was soon found that Hopkins' reagent may mean any one of three types of solution: that which contains 10 per cent mercuric sulfate in 7 per cent by volume sulfuric acid, that which contains 10 per cent mercuric sulfate in 5 per cent by volume sulfuric acid, and, possibly, that which contains 10 per cent mercuric sulfate in 5 per cent by weight sulfuric acid. This led to a study of the precipitation of pure histidine with mercuric sulfate in different concentrations of sulfuric acid. The results, which are reported in Table IV, are quite striking.

It was purely by chance that the directions of Cole (20) for the preparation of Hopkins' reagent were followed; thus, 7 per cent sulfuric acid by volume was used. In Experiments 8 and 9 in Table IV further dilution with an equal volume of water gave 0.42 per cent and 0.43 per cent histidine nitrogen, respectively, making the total recovery in these two experiments 94.22 and 94.03 per cent. This was the maximum recovery in any experiment. Further recovery could not be obtained by making the solutions more dilute. In all experiments the recovery was above 90 per cent when the acid concentration was brought to approximately 4 or 5 per cent by weight.

Since the concentration of sulfuric acid so markedly influenced the solubility of the mercury salt of histidine, there may be other factors which will affect it also. This was indicated by the specific

directions of Vickery and Leavenworth (1) for the complete removal of chlorides before precipitation with Hopkins' reagent, although they made no comment on this procedure. A study of this point has not been reported in the literature, but a preliminary investigation indicated that chlorides did interfere. In one experiment with histidine dichloride no precipitate formed in 48 hours, but 55 per cent was precipitated in 120 hours. In another experiment in which a more concentrated solution of histidine dichloride was used 76 per cent was recovered in 48 hours. In similar experiments in the absence of chlorides over 90 per cent should have been expected as shown in Table IV. However, one investigator has made use of mercuric chloride in a quantitative method for the estimation of histidine, although he does not discuss the reasons for the choice of this reagent.

There can be no doubt, however, that the material precipitated from the crude histidine fraction by Hopkins' reagent consisted chiefly of histidine since most of the nitrogen precipitated could be accounted for by the histidine flavianate which was isolated. Analyses of several samples of the histidine flavianate showed that it was quite pure.

From the data obtained in this investigation, it is evident that the amount of histidine present in casein is still unknown, for it is doubtful whether the amount isolated as the flavianate represents the true value. The same is true for the histidine content of edestin as Vickery and Leavenworth (1) have already stated.

*Estimation of Lysine.*—The only methods that have been extensively used for the estimation of lysine are the indirect method of Van Slyke (6) and the direct isolation as the monopicrate. The first method has consistently yielded higher results than the second. The Van Slyke method for the lysine of casein usually gives values which are above 10 per cent. The values obtained by Leavenworth (21) are probably the highest values ever obtained by isolation. Working with large quantities of casein, using as much as a 2 kilo sample in one experiment, and reworking the mother liquors, he was able to isolate 5.77 per cent of lysine or 7.37 per cent of lysine nitrogen. He stated that this value probably closely approached the maximum amount of lysine present in casein. The technique described in the first part of this paper gave values which approach the value of Leavenworth as

closely as could be expected, when small quantities of protein are being worked with and if the amount that remains in the mother liquors is disregarded. The amount of lysine isolated both by Leavenworth (21) and in this investigation is far below the total nitrogen of the lysine fraction.

### SUMMARY.

1. The modified method of Vickery and Leavenworth (1) for the estimation of the basic amino acids of protein has been further modified for use on small amounts of protein. Several changes were found advisable and the complete details are reported.

2. The basic amino acids of casein and edestin were determined by this method with very consistent results. The values obtained for edestin correspond very closely with those reported by Vickery and Leavenworth, and the values for the lysine content of casein correspond well with that obtained by Leavenworth (21) when very large quantities of casein are used.

3. The histidine and arginine content of casein have also been estimated by other methods for comparison.

4. The total nitrogen of the arginine fraction is not a true indication of the amount of arginine present, while the alkaline hydrolysis method of Van Slyke (6) probably is a true indication. The isolation method gives values corresponding closely with those obtained by alkaline hydrolysis.

5. The colorimetric method of Hanke and Koessler (3) and the bromination method of Plimmer and Phillips (5) give values which agree well with the values obtained for the total nitrogen of the histidine fraction.

6. The bromination method of Plimmer and Phillips is accurate only under very special conditions. It is influenced by temperature, acid concentration, and length of time of bromination, but most notably by temperature. There are also several interfering substances.

7. The solubility of the mercury compound of histidine is markedly influenced by the concentration of acid, since it will not precipitate from a sulfuric acid solution when the acid concentration is above 13 per cent by weight, but it is almost quantitatively precipitated when the acid concentration is between 3 and 5 per cent by weight.

8. The results obtained in this investigation indicate that the modified method described can be applied to small amounts of protein with a considerable degree of accuracy.

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# SOME CHEMICAL INVESTIGATIONS OF EMBRYONIC METABOLISM.

## IV. AN INVESTIGATION OF THE BASIC AMINO ACIDS OF THE HEN'S EGG DURING DEVELOPMENT.*

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In the preceding paper of this series (1) a résumé was given of the investigations by various workers of the changes in some of the amino acids and other nitrogenous constituents of the hen's egg during development. In the same publication a report was made of a complete study, by the Van Slyke distribution method, of the changes which occur in the various forms of nitrogen. This was a study of the daily changes which occur over the entire period of development of 21 days.

The present investigation was undertaken because it seemed desirable to have more accurate information concerning the behavior of the basic amino acids than could be obtained by the Van Slyke method. The basic amino acids, especially arginine and histidine, have often been considered precursors of creatine and the purines. In the developing egg we are dealing with a closed system for 21 days, almost, if not entirely, free from bacteria, and in which very marked chemical changes occur. The system is entirely uncomplicated by dietary considerations, and the only exchanges with the surroundings that occur are a loss of moisture and carbon dioxide and absorption of oxygen. If a certain amino acid remains constant in amount during the period of development, it is almost certain that it is not being used as a precursor. While if an amino acid decreases, it is just as certain that it is being transformed into other substances with which in

some cases it may be structurally related.   The problem is complicated by the fact that the changes may be very small and thus easily escape detection.

The methods used in this work have been the author's modification of the method of Vickery and Leavenworth (2) reported in another paper (3), the colorimetric method of Hanke and Koessler (4), and the bromination method of Plimmer and Phillips (5) for histidine and the alkaline hydrolysis method of Van Slyke (6) for arginine, with the modification of the method suggested by Plimmer (7) and the Koehler (8) modification of the apparatus. As pointed out in Paper III (1) of this series the accuracy of the Van Slyke method for arginine, histidine, and lysine has been questioned many times.   However, the author's modified method previously described has been used in the present study of the developing hen's egg with very consistent and apparently quite accurate results.

<div align="center">EXPERIMENTAL.</div>

The material in this investigation was part of the same material used for the nitrogen distribution study previously reported (1). The number of eggs used and the preparation of the material were described in detail in that paper.   The results obtained by the various methods are recorded in Tables I and II.   The values are expressed in percentage of the total nitrogen present in the solution after hydrolysis.   Some analyses of arginine flavianate and histidine flavianate obtained at different periods in this investigation are reported.   The purity of the lysine picrate was satisfactory as indicated by the melting point.

<div align="center">*Arginine Flavianate.*</div>

Micro-Dumas-Pregl for nitrogen (9).
    1st day.   3.186 mg. gave 0.488 cc. N at 24° and 744 mm.
20th   "      3.284  "     "   0 504  "  "  "  24°  "   742   "
Required for $C_6H_{14}N_4O_2 \cdot C_{10}H_6N_2SO_8$. . . . . . . . . . . . .. . .... . N 17 21
Found, 1st day. .. . . . . . . . . . . . . . . . . . . . . . .  . . . . . . . . . . . . . ..." 17 22
        20th   "   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . ." 17 25

<div align="center">*Histidine Flavianate.*</div>

Micro-Dumas-Pregl for nitrogen (9).
Fresh egg.   4 287 mg. gave 0 479 cc. N at 23° and 738 mm.
20th day.   4 274  "     "   0 478  "  "  " 23°  "   738   "

Required for $C_6H_9N_3O_2 \cdot 2C_{10}H_6N_2SO_6$.....................N 12.52
Found, fresh egg........... ............................" 12.52
   20th day. ......................................" 12.56

## DISCUSSION.

In Paper III of this series a discussion of the differences in the results obtained in that investigation as compared with those of other investigators was purposely omitted. It seemed best to delay the discussion until the results of the present investigation were available.

In view of the many criticisms of the Van Slyke method and in view of the fact that Van Slyke stated that several substances, among them the purines, interfere, it is evident that the values obtained for cystine, arginine, histidine, and lysine in the developing hen's egg by this method are not accurate and may not even be consistent, since at the beginning of the developmental period the nitrogenous constituents consist more nearly of pure protein than at the end of the period. It has been shown by a number of workers that the purines, especially, increase during development. The basic amino acids have been estimated by the Van Slyke distribution method in the preceding investigation of this series by the author and reported (1) and by Plimmer and Lowndes (10). The values found in these two investigations agree quite well with each other, but do not agree with those of the present extended study or with those of other investigators.

It is a striking fact that there were practically no changes observed in the arginine content of the developing hen's egg as determined by all of these methods, except on the last day or two when an increase was observed. The arginine values obtained by the Van Slyke distribution method previously reported, and included in Table I, showed a slight decrease in the middle of the period and an increase at the end. The increase at the end of the period, as indicated by three of the four methods, cannot be explained. The slight decrease during development found by Sendju (11) can be attributed to the method, since he estimated the bases precipitated by phosphotungstic acid and, as previously stated, this method has been shown to be inconsistent. However, the tremendous decrease of 18 per cent of the arginine reported by Russo (12) cannot be so easily explained. The method was not

stated in the original paper, but it was probably the modification of the Van Slyke method proposed by Russo (13).    These results are all the estimations of arginine in the whole egg that have been reported.    Plimmer and Lowndes did not include the shell, and

TABLE I

*Arginine and Lysine Content of Developing Hen's Eggs*

The values are expressed in per cent of total N

| Period of incubation | Sample | Arginine N | | | | Lysine N | | |
|---|---|---|---|---|---|---|---|---|
| | | N, Van Slyke distribution method | Total N in arginine fraction | N, alkaline hydrolysis, Van Slyke | Isolation as flavianate | N, Van Slyke distribution method | Total N in lysine fraction | Isolation as picrate |
| *days* | *gm* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Fresh egg | 50 | 14 63 | 11 04 | 9 90 | 9 48 | 8 40 | 10 90 | 7 04 |
| 1 | 5 | 14 36 | 10 84 | 9 84 | 9 55 | 8 00 | 9 33 | 6 71 |
| 2 | 5 | 14 18 | 11 36 | 9 68 | 9 65 | 8 05 | 9 65 | 5 89 |
| 3 | 5 | 13 55 | 10 29 | 9 89 | 9 46 | 9 35 | 10 43 | 6 34 |
| 4 | 5 | 14 27 | 10 43 | 9 31 | 8 87 | 8 26 | 10 87 | 6 02 |
| 5 | 25 | 14 74 | 9 93 | 9 86 | 9 63 | 11 00 | 8 86 | 6 40 |
| 6 | 5 | 14 35 | 10 37 | 9 72 | 9 33 | 8 91 | 8 96 | 6 42 |
| 7 | 5 | 14 10 | 11 09 | 9 36 | 8 59 | 8 95 | 8 81 | 6 25 |
| 8 | 5 | 14 12 | 10 73 | 9 43 | 9 27 | 9 70 | 9 10 | |
| 9 | 5 | 14 30 | 11 32 | 9 83 | 9 56 | 9 55 | 8 88 | 6 15 |
| 10 | 25 | 13 25 | 11 63 | 9 58 | 9 59 | 8 53 | 8 60 | 5 78 |
| 11 | 5 | 13 40 | 10 88 | 9 29 | 9 18 | 8 40 | 8 57 | 5 56 |
| 12 | 5 | 13 87 | 9 98 | 9 38 | 9 29 | 9 10 | 8 92 | 5 45 |
| 13 | 5 | 13 88 | 11 12 | 9 47 | 9 31 | 9 63 | 8 63 | 5 28 |
| 14 | 5 | 13 78 | 10 24 | 9 33 | 9 11 | 10 48 | 8 44 | |
| 15 | 25 | 13 32 | 12 66 | 9 33 | 9 53 | 9 60 | 9 10 | 5 05 |
| 16 | 5 | 13 92 | 10 76 | 9 62 | 9 46 | 9 91 | 8 73 | 4 78 |
| '17 | 5 | 14 42 | 11 33 | 9 48 | 9 26 | 9 76 | 9 71 | 4 61 |
| 18 | 5 | 15 28 | 10 68 | 9 87 | 9 63 | 8 92 | 8 92 | |
| 19 | 5 | 15 70 | 11 21 | 9 69 | 9 43 | 10 12 | 9 20 | 4 33 |
| 20 | 25 | 15 82 | 12 84 | 9 91 | 9 83 | 10 12 | 8 30 | 4 06 |
| 21 | 25 | 16 46 | 12 48 | 10 50 | 9 80 | 8 36 | 6 52 | 3 21 |

one is unable to tell whether Sendju (11) and Russo (12) included the shell in their work, or only the contents of the shell.

In the course of this investigation an unsuccessful attempt was made to determine the arginine by the direct method proposed by Plimmer and Rosedale (14).    The values for arginine ranged

from 24.22 per cent to 35.08 per cent for the same day. Several estimations were made, but the values were seldom duplicates and always even higher than those obtained by the Van Slyke distribution method. The isolation values for arginine as flavianate, although the lowest, are probably the most accurate of all.

TABLE II.

*Histidine Content of Developing Hen's Eggs.*

The values are expressed in per cent of total N.

| Period of incubation. | Sample. | N, Van Slyke distribution method. | Total N in histidine fraction. | Bromination, Plimmer and Phillips. | Colorimetric, Hanke and Koessler. | N precipitated by mercuric sulfate, Kossel and Patten. | Isolation as flavianate. |
|---|---|---|---|---|---|---|---|
| *days* | *gm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Fresh egg. | 50 | 2.31 | 5.86 | 6 70 | 5.03 | 3.75 | 3.81 |
| 1 | 5 | 3.10 | 5.68 | 5.83 | 4.98 | 3.92 | |
| 2 | 5 | 3.36 | 6.07 | 5.87 | 4.73 | 3.82 | |
| 3 | 5 | 2.40 | 6.02 | 5.78 | 4.70 | 3.99 | |
| 4 | 5 | 3.46 | 6.95 | 6.13 | 4.53 | 3.71 | |
| 5 | 25 | 1.36 | 6.07 | 5.83 | 4.93 | 3.76 | 3.68 |
| 6 | 5 | 3.06 | 6.18 | 6.23 | 4.63 | 3.58 | |
| 7 | 5 | 1.98 | 5.82 | 5.92 | 4.38 | 4.47 | |
| 8 | 5 | 1.98 | 6.18 | 5.87 | 4.32 | 3.83 | |
| 9 | 5 | 2.32 | 6.32 | 6.12 | 4.35 | 3.41 | |
| 10 | 25 | 4.74 | 6.14 | 5.90 | 4.42 | 3.79 | 3.51 |
| 11 | 5 | 3.23 | 6.23 | 5.92 | 4.26 | 3.51 | |
| 12 | 5 | 1.12 | 6.26 | 5.61 | 4.23 | 3.43 | |
| 13 | 5 | 3.64 | 6.28 | 6.03 | 4.18 | 3.35 | |
| 14 | 5 | 1.35 | 6.23 | 5.93 | 4.21 | 3.17 | |
| 15 | 25 | 3.01 | 6.25 | 6.00 | 4.00 | 3.13 | 3.13 |
| 16 | 5 | 2.10 | 6.42 | 6.07 | 3.71 | 2.96 | |
| 17 | 5 | 2.09 | 6.67 | 5.98 | 3.65 | 3.01 | |
| 18 | 5 | 2.76 | 6.73 | 5.89 | 3.41 | 2.70 | |
| 19 | 5 | 1.08 | 6.85 | 6.12 | 3.50 | 2.56 | |
| 20 | 25 | 1.69 | 7.68 | 6.01 | 3 41 | 1.35 | 1.38 |
| 21 | 25 | 1.81 | 7.98 | 5.87 | 2.68 | 1.42 | 1.26 |

A consideration of the values reported for histidine in Table II leads to the conclusion that the amount of histidine present in the hen's egg is not definitely known. It may be significant that the values obtained by isolation as the flavianate were of the same order as those obtained by the Van Slyke distribution method

which usually gives higher values for histidine than other methods. The values obtained by the Hanke and Koessler method (4) were about 1 per cent higher throughout than those obtained by isolation, but showed exactly the same relative changes.   In spite of the fact that the values for total nitrogen of the histidine fraction showed a slight gain and that the bromination values showed no change, the author believes that the actual amount of histidine present in the hen's egg decreases during the period of development.   Plimmer and Lowndes (10) stated that the histidine values obtained in their investigation were meaningless, which was quite evident from their results.   Sendju (11) found a definite increase in histidine, while Russo (12) found a very slight decrease. These are the only investigations of the histidine content of the whole egg during development that have been reported.

There were no changes indicated in the lysine when the Van Slyke distribution method was used, which was not in agreement with the fact that the isolation method showed a marked decrease as indicated in Table I.   A decrease in the amount of lysine present is what would be expected if it is used in the destructive or precursor sense; that is, if it is being broken down and the breakdown products are used to build other substances.   However, it may be used simply as a building stone for the new tissues being formed by the embryo; that is, it may be transferred from the various parts of the egg to the embryo and used as such in the synthesis of tissue proteins.   Neither theory has ever been proved and the lysine is probably used for both purposes.   The values obtained by Plimmer and Lowndes (10), by Sendju (11), and by Russo (12) show practically no changes during the period of development of the hen's egg.

The findings reported in this paper agree in general with the few reported by Plimmer and Lowndes (10) and with those of Sendju (11), but not with those of Russo (12) who found an 18 per cent decrease in the basic amino acids, nearly all of which could be attributed to the loss in arginine.   The methods used in this investigation for the arginine all show that the arginine remains practically constant.   However, in the case of histidine the methods gave values which were very inconsistent.   One showed a slight increase, one showed no change, while all others showed a marked decrease.   It is believed that those methods which show a

decrease in the histidine represent the true facts. Both arginine and histidine have been thought to be precursors of purines (15), but more recent work seems to show that only histidine is used as a precursor of purines (16). The results of this investigation support the latter theory since arginine does not decrease and most methods show that the histidine does decrease during the development of the hen's egg.

The methods for the estimation of lysine have been inconsistent, also, in that some show no change in the lysine content, while the isolation method shows a decrease in the lysine during the period of development.

<div align="center">SUMMARY.</div>

1. The basic amino acids of the developing hen's egg have been determined by the modified method previously described (3) and by other methods throughout the entire period of development.

2. The arginine remains practically constant during the entire period. The values obtained by isolation as the flavianate probably more nearly represent the actual amount of arginine present than do those obtained by the Van Slyke distribution method which are much higher.

3. The methods for the estimation of histidine gave inconsistent results. One showed a slight increase, one showed no change, while all others showed a marked decrease, and it is believed that histidine does decrease during the period of development.

4. The values obtained for lysine were also inconsistent, but it is believed that the isolation values more nearly represent the true facts and that the lysine decreases during the development of the hen's egg.

5. The results obtained in this investigation are entirely in accord with the theory that arginine does not serve as a precursor of purines, while histidine may do so.

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# THE QUESTION OF THE OXIDATION OF GLUCOSE IN PHLORHIZIN GLYCOSURIA.

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It has recently been shown by the studies of Wierzuchowski (22) and of Deuel, Wilson, and Milhorat (8) that the administration of glucose to completely phlorhizinized fasting dogs causes a definite rise in the respiratory quotient which may persist for as long as 4 hours after administration. Wierzuchowski (23) also observed a reduction in the ketosis of the blood and urine with a concomitant rise in the carbon dioxide capacity of the blood and he recovered, on the average, about 82 per cent of the ingested sugar in the urine. All these investigators concluded that in phlorhizin diabetes the organism is still able to oxidize approximately one-fifth or one-sixth of the ingested glucose. In some of the experiments of Deuel, Wilson, and Milhorat the amount of glucose accounted for by oxidation added to that calculated as "extra glucose" in the urine slightly exceeded the amount of glucose administered.

The present experiments were undertaken first, to see what proportion of the ingested sugar could be accounted for in completely phlorhizinized dogs by the rise in respiratory quotient (on the assumption that this indicated an increased rate of oxidation of carbohydrate) and second, to determine whether this amount added to the extra glucose of the urine would or would not exceed the quantity of sugar administered beyond the limits of experimental error. We also confirmed the well known effect of phlor-

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hizin in raising the basal heat production and have observed, in
addition, that when glucose is administered under these conditions
there is a slight but definite decrease from the abnormally high
heat production. The problem of ascertaining quantitatively
the fate of glucose administered to phlorhizinized dogs depends
even more on the validity of the D:N ratio than it does on the
respiratory quotient, as the quantity of carbohydrate oxidized is
approximately only one-fifth of that excreted. Therefore we have
discussed our data quite fully in regard to the experimental condi-
tions which must be observed to obtain a D:N ratio which would
most accurately represent the carbohydrate derived from protein
and which would avoid as far as possible the errors due to a
variation in the carbohydrate stores during the experimental
period.

The animal on which the first four respiratory studies were
made was a healthy, well trained, female dog, weighing approxi-
mately 14 kilos, which had been used in metabolism work for
several years. It was accustomed to the various procedures
and its basal level of heat production was known to have remained
very close to 18 calories per hour for 2 years. For several weeks
before the present study was begun the dog had been on a standard
maintenance diet consisting of 230 gm. of a mixture containing
44 per cent fat-free beef heart, 44 per cent cracker meal, 8 per cent
lard, and 4 per cent bone ash. During the interim between
Experiments 1 and 2 extra carbohydrate in the form of Karo corn
syrup was given to favor recovery from the effect of phlorhizina-
tion; this, however, was omitted in the subsequent intervals
because we suspected, as will be shown later, that in Experiment
2 this might have been the cause of the apparent recovery of
more glucose than had been administered. With this exception
all food was stopped for 1 or 2 days before phlorhizin was adminis-
tered. The initial dose of phlorhizin was 2 gm. subcutaneously,
1 gm. being administered in 10 cc. of olive oil and the other in
10 cc. of 1 per cent sodium carbonate solution; on the succeeding
days 1 gm. of phlorhizin in olive oil was given each morning until
the day of the glucose test when, except in Experiment 1, an addi-
tional gm. of phlorhizin was given in the sodium carbonate solution.
In an attempt to reduce the glycogen and carbohydrate stores
to a minimum in Experiments 1 to 4 the animal was given a cold

bath on the 2nd day after the phlorhizin was begun and then kept in a cool place for 5 hours so that definite shivering was induced in all cases. In Experiment 1 a single dose of 1 cc. of 1:1000 epinephrine was administered intramuscularly on the day preceding the administration of glucose as an additional measure to restore the carbohydrate reserves; in Experiment 5 frequent repeated doses of epinephrine were used, as will be described in detail later.

The respiratory metabolism was determined by the gasometer method according to the technique described by Boothby and Sandiford (2) and adapted to animal studies by Kitchen (10). Briefly, the method consists in a nearly continuous collection of the expired air in a gasometer for 15 minute periods with 5 minute intervals between individual tests. Duplicate analyses of aliquot samples of the expired air, collected over mercury, are then made for the percentage of carbon dioxide and oxygen with the Haldane gas analysis apparatus. There are several advantages in the gasometer or open circuit method of an indirect calorimeter as compared with the closed circuit respiration calorimeter, for this type of experiment, which offset the disadvantage of not having as a control the direct calorimetric measurement. These advantages are: first, the actual basal metabolism can be determined just before the administration of the glucose; second, the glucose can be given either intravenously or through a gastrostomy opening without interruption of the respiratory experiment and without causing any disturbance to the dog, and third, the immediate effect of the glucose on the respiratory quotient and heat production can be accurately determined, thus avoiding the necessity of assuming any values during the 1st hour after its administration. To study the effect of glucose 16 gm. were dissolved in 100 cc. of distilled water and administered at approximately body temperature; in Experiments 1 and 2 it was given through a permanent gastrostomy opening, and in Experiments 3, 4, and 5 it was given intravenously; the method of administration did not demonstrably affect the result. A series of four to six satisfactory basal tests was obtained immediately before the glucose was given. The urine was collected throughout the entire phlorhizination period; the animals were catheterized each morning and, on the days of glucose administration, again at the end of the respiratory experiment; while the animal was in the metabolism

cage the urine was collected in a vessel containing thymol.   The
total nitrogen was determined by the Kjeldahl method and the
sugar by the Benedict method.   In calculating the diabetic non-
protein respiratory quotients we have employed the method of
Lusk (12) in which the calculation is based on the quantity of
glucose and nitrogen excreted in the period just preceding the
administration of glucose.
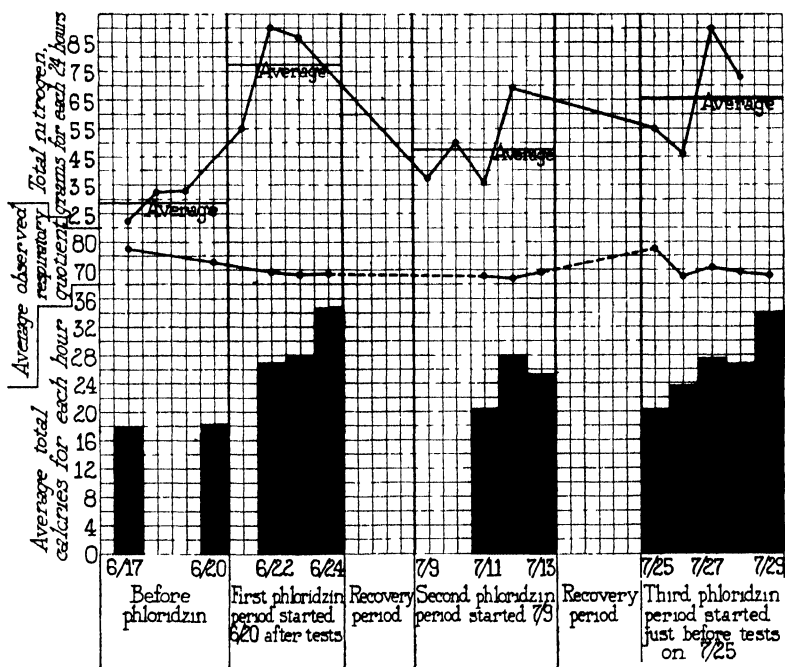


Fig 1  The effect of phlorhizination on the basal heat production, the
observed respiratory quotient, and nitrogen excretion.

The increase on basal heat production which occurs during the
course of complete phlorhizination is shown in Fig. 1.   In Figs. 2
and 3 and in Tables I to III are given the results of the respiratory
data following the administration of glucose in Experiments 1
to 5.   The individual determinations in Experiments 1 and 5 have
been plotted in Figs. 2 and 3 but for the other experiments, to
conserve space, the results of the individual tests falling within a

given hour have been averaged and expressed in the tables on the hourly basis. The urinary data are given in Tables IV to VII.

The injection of 1 or 2 gm. of phlorhizin in olive oil or sodium carbonate solution is not followed by an immediate rise in heat production; by the next morning, however, there is usually a definite increase which thereafter rapidly becomes marked so that after several days on phlorhizin the average basal heat production may be, as shown in Fig. 1, nearly 90 per cent above the normal basal value. These data confirm the previous observations of

TABLE I.

*Respiratory Data, Experiment 2, July 12, 1927.*

| Hr | Total O₂ consump- tion | Non-protein R Q | Carbo- hydrate | Total calories | Comments. |
|---|---|---|---|---|---|
| | *l per hr* | | *calories* | | |
| Basal | 6 05 | 0 675 | 0 0 | 27 9 | Quiet    16 gm glu- cose  by  gastros- tomy     at     10 00 a m |
| 1st | 5 44 | 0 717 | 1 0 | 25 1 | Quiet |
| 2nd | 5 12 | 0 714 | 0 2 | 23 5 | " |
| 3rd | 5 05 | 0 750 | 3 1 | 23 4 | " |
| 4th | 4 64 | 0 728 | 1 5 | 21 4 | " |
| 5th | 4 67 | 0 736 | 2 2 | 21 5 | " |
| 6th | 4 79 | 0 724 | 0 9 | 22 1 | " |
| 55 min | 4 85 | 0 742 | 2 2 | 22 9 | Restless |
| | | | 11 1 = 3 1 gm  glucose oxidized | | |

Protein calories = 3 6 each hour; calculated on a total nitrogen excretion of 0 27 gm each hour and a D N ratio of 3 53.1.

Lusk (12) who obtained an elevation of basal heat production of 70 per cent above the normal value.

In each of our experiments, as illustrated in Fig. 2, the administration of glucose to the completely phlorhizinized animal resulted in a definite and marked decrease in heat production. In the five experiments this decrease, during the period in which the respiratory metabolism was determined, amounted to 6, 33, 8, 13, and 11 calories respectively, an average reduction of approximately 10 per cent following the administration of glucose. This effect of glucose in phlorhizin glycosuria, although occasionally

recorded in the data of previous investigators, has not been emphasized. In some of Lusk's (11) tables definite decreases in heat production are seen; for instance, after 10 gm. of glucose, the heat production decreased from a previous value of 23.8 calories per hour to 20.4 calories per hour in the 2nd hour after the adminis-
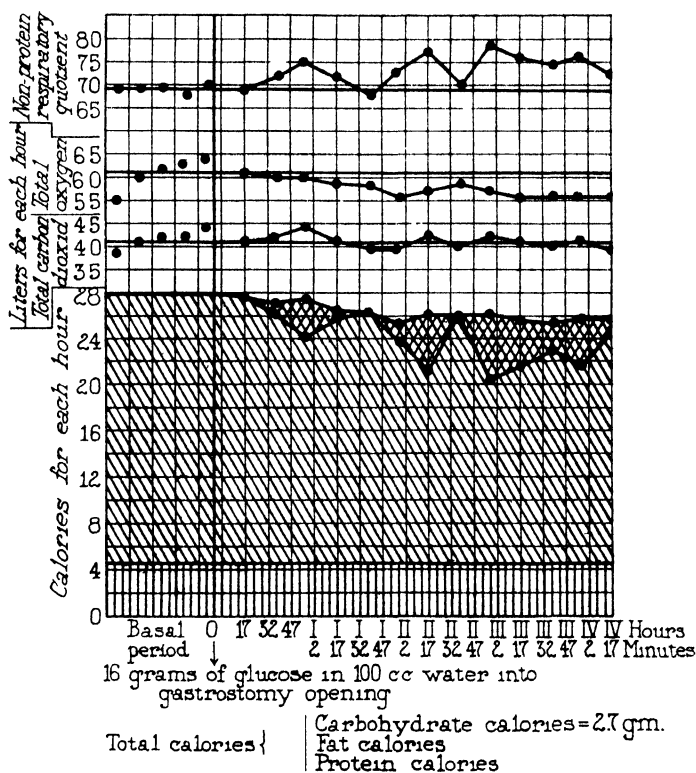


Fig. 2 The effect of the administration of 16 gm of glucose to a phlorhizinized dog

tration of glucose, and in another set of experiments following the ingestion of 10 gm. of fructose the metabolism dropped from 29.4 to 24.8 calories per hour in the 4th hour after ingestion in one experiment, and from 27.1 to 24.8 in the second experiment. The protein-sparing action of glucose in phlorhizin diabetes has been

clearly demonstrated by the work of Ringer (17, 18), Deuel and Chambers (7), Deuel, Wilson and Milhorat (8), and Wierzuchowski (22, 23), and it is possible that this effect of glucose may bear some relation to the decrease in heat production which we have observed. We were unable, however, to establish any mathematical relationship between the quantity of glucose apparently oxidized, the decrease in nitrogen elimination, and the decrease to a lower but still abnormally high level of heat production. Wierzuchowski found that the reduction of the ketosis and the decrease in nitrogen elimination were proportional to the amount

TABLE II.

*Respiratory Data, Experiment 3, July 28, 1927.*

| Hr. | Total $O_2$ consumption | Non-protein R Q. | Carbo-hydrate | Total calories. | Comments. |
|-----|------|------|------|------|-----|
| | *l per hr* | | *calories* | | |
| Basal. | 5 70 | 0 702 | 0 0 | 26 2 | Quiet.  16 gm. glucose intravenously at 10.42 a.m. |
| 1st | 5 58 | 0 766 | 5 2 | 25 9 | Quiet. |
| 2nd | 5 03 | 0 746 | 3 2 | 23 1 | " |
| 3rd | 5 00 | 0 754 | 3 1 | 23 0 | " |
| 4th | 5 38 | 0 724 | 1 0 | 24 7 | " |
| 5th | 5 92 | 0 707 | 0 0 | 27 1 | " |
| | | | 12 5 = 3.4 gm glucose oxidized. | | |

Protein calories = 2 9 each hour; calculated on a total nitrogen excretion of 0.28 gm. each hour and a D:N ratio of 4.33:1.

of glucose given and not to the unrecovered portion. The clearing up of the acidosis may also have been a factor in the decrease in heat production following glucose. The relatively small decrease in heat production of 10 per cent following the administration of glucose, with the coincident decrease in nitrogen elimination and reduction of ketosis indicates that the large rise in heat production in phlorhizinized dogs, amounting in some instances on the 4th or 5th day to 90 per cent, cannot be explained entirely by either of these factors or even by a summation of their effect; some other factor as yet unknown must, therefore, be the cause of the major part of the increased heat production.

The rise in respiratory quotient was evident 15 to 30 minutes after glucose was given and it reached a maximum of between 0.75 and 0.85 in all experiments. The elevation may persist with irregular fluctuations until the end of the 7th hour. If we assume that the rise in respiratory quotient indicates oxidation of carbohydrate, then it may be calculated, without allowance for retained carbon dioxide, that the glucose oxidized amounted, respectively, to 2.7, 3.1, 3.4, 2.2, and 7.1 gm. in the five experiments. This is an average of 2.9 gm. or approximately 18 per cent of the amount administered in Experiments 1 to 4; the larger amount apparently oxidized in Experiment 5 may be due to the large amount of

TABLE III.

*Respiratory Data, Experiment 4, July 29, 1927.*

| Hr. | Total O₂ consumption. | Non-protein R Q | Carbohydrate. | Total calories. | Comments |
|---|---|---|---|---|---|
| | *l. per hr.* | | *calories* | | |
| Basal. | 7 34 | 0 695 | 0 0 | 33 8 | Quiet. 16 gm. glucose intravenously at 10.00 a.m. |
| 1st | 6 94 | 0 749 | 4 5 | 32 3 | Quiet. |
| 2nd | 6 50 | 0 728 | 2 2 | 30 0 | " |
| 3rd | 6.32 | 0 718 | 1 2 | 29 1 | " |
| 4th | 6.47 | 0 710 | 0 3 | 29 8 | " |
| | | | | 8 2 = 2.2 gm. glucose oxidized. | |

Protein calories = 3.3 each hour; calculated on a total nitrogen excretion of 0.30 gm. each hour and a D:N ratio of 4.23:1.

epinephrine given during the preliminary periods. If the extreme allowance for retained carbon dioxide referred to in detail in the following paragraph is made, this figure would average 3.7 gm. or 23 per cent of the amount ingested. In the experiments of Deuel, Wilson, and Milhorat the amount of glucose oxidized (without allowing for changes in carbon dioxide-combining power) averaged 19 per cent of the 16 gm. given; in the two experiments of Wierzuchowski, in which the acidosis had been abolished by the previous administration of glucose, the amount oxidized averaged 17 per cent of the 20 gm. given and 12 per cent in the experiment in which this precaution had not been taken. In all of the respira-

tory experiments the basal non-protein respiratory quotient before the administration of glucose was between 0.675 and 0.702 except in the last experiment shown in Fig. 3.   In Experiment 5 the basal non-protein respiratory quotients averaged 0.73 but were irregular.   The dog also showed signs of great exhaustion and evidence of hypoglycemia as a result of the administration of a large amount of epinephrine; the breathing was also irregular, suggesting the possibility that there were periods of temporary overventilation.

TABLE IV.
*Urinary Data, Experiment 1.*

| Date | Total N | Sugar | D N ratio | Comments. |
|------|---------|-------|-----------|-----------|
| *1927* | *gm* | *gm* | | |
| June 19–20 | 3 30 | | | Last feeding 8 30 a m , June 19. |
| "     20–21 | 4 26 | 18 7 | 4 39 | 2 gm phlorhizin |
| "     21–22 | 5 48 | 17 7 | 3 23 | 1 "        "        shivered 5 hrs. |
| "     22–23 | 9 16 | 35 3 | 3 85 | 1 "        "        1 cc  epinephrine.* |
| "     23–24 | 8 74 | 43 7 | 5 00 | 1 "        "        16 gm  glucose |

|  | *gm.* |
|---|---|
| Total glucose excreted | = 43 7 |
| Glucose from protein = 8 74 × 3 85 | = 33 7 |
| "Extra" glucose recovered in urine | = 10 0 |
| Glucose oxidized (Fig  2) | = 2 7 |
| "       accounted for | = 12 7 |

* Epinephrine given June 22 and caused slight rise in D:N ratio.   The D N ratio of 3 85 which is used to calculate the extra glucose was obtained on the morning of June 23, just before the injection of glucose.

We observed the carbon dioxide capacity of the blood in another dog similarly prepared and given 16 gm. of glucose by stomach tube.   The carbon dioxide capacity of the whole blood gradually rose 9.6 volumes per cent (from 34.7 to 44.3) in 3 hours, or an average of 3.2 volumes per cent each hour, which is essentially the same as found by Wierzuchowski.   He obviated the error in the respiratory quotient due to the change in the carbon dioxide-combining power by giving a preliminary dose of glucose which reduced the ketosis and restored the carbon dioxide capacity of the blood almost to normal; the results of a second dose of glucose,

given 4 hours later, were then only slightly influenced by shifts in carbon dioxide capacity of the blood. This we did not do but have made the following calculations to determine the possible
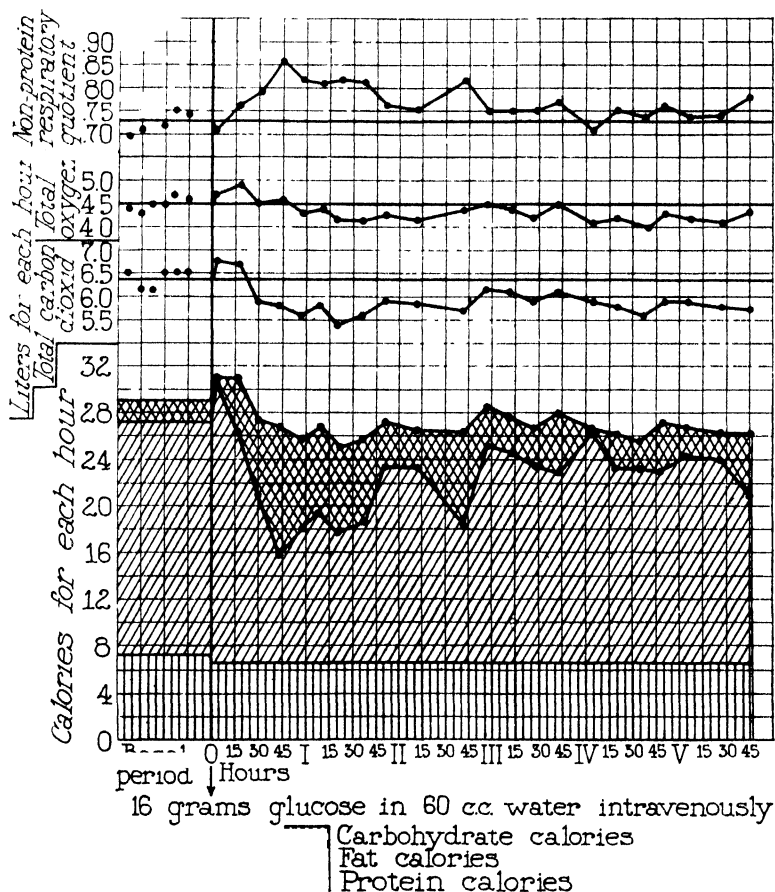


FIG. 3. The effect of 16 gm. of glucose when administered to a dog in which the carbohydrate reserves had been reduced to a minimum by repeated injections of epinephrine.

effect of a similar increase in carbon dioxide capacity of the blood on the non-protein respiratory quotient during the 1st hour after the administration of glucose in Experiment 1. In these calcula-

tions we have assumed that the increase in carbon dioxide capacity of the blood is accompanied by a concomitant and parallel decrease in hydroxybutyric acid, as shown by Wierzuchowski, so that for every 44 gm. of carbon dioxide retained, 104 gm. of hydroxybutyric acid were oxidized. The oxygen needed to oxidize the calculated amount of hydroxybutyric acid was subtracted from the total non-protein oxygen and the carbon dioxide resulting from the oxidation of the hydroxybutyric acid minus the carbon dioxide retained in the body to neutralize the freed base was subtracted from the

TABLE V.

*Urinary Data, Experiment 2*

| Date | Total N. | Sugar | D N ratio. | Comments |
|---|---|---|---|---|
| *1927* | *gm* | *gm.* | | |
| July 8–9 | 3 09 | | | Last feeding 11 00 a.m , July 8. |
| " 9–10 | 3 70 | 32 3 | 8 72 | 2 gm phlorhizin. |
| " 10–11 | 5 00 | 27 4 | 5 54 | 1 "        " |
| " 11–12 | 3 63 | 12 8 | 3 53 | 1 "        "        shivered 5 hrs. |
| " 12–13 | 6 94 | 41 3 | 5 95 | 2 "        "        16 gm. glucose. |

|  | *gm.* |
|---|---|
| Total glucose excreted | 41 3 |
| Glucose from protein = 6 94 × 3 53 | = 24 5 |
| "Extra" glucose excreted | = 16 8 |
| Glucose oxidized | = 3 1 |
| "        accounted for | = 19 9* |

* The large amount of glucose accounted for in this experiment may possibly be due to the fact that in the interim between this and the preceding experiment in addition to the standard maintenance diet the dog was given carbohydrate in the form of Karo corn syrup.

total non-protein carbon dioxide; the resulting respiratory quotient may be assumed as indicating the metabolic quotient. In one calculation we considered only the changes in the blood (estimated as 6 per cent of the body weight) and found that the change in the non-protein respiratory quotient would be negligible; that is, from 0.721 to 0.722. In a second calculation we assumed first, that equilibrium changes were going on in the soft tissues of the body as a whole, second, that these tissues had the same average degree of buffer action as the blood, and third, that the mass of soft tissues in equilibrium with the blood is about 60 per cent of

the body weight. On these assumptions the non-protein respiratory quotient was elevated from 0.721 to 0.742 during the 1st hour, with a similar increase in the 2 following hours. It is thus seen that the maximal allowance for readjustment of the acid-base equilibrium with carbon dioxide retention would increase the regularly calculated value of carbohydrate combustion, as shown in Tables I to III, by approximately 0.8 gm. of carbohydrate,

TABLE VI.

*Urinary Data, Experiment 3.*

| Date. | Total N. | Sugar. | D:N ratio. | Comments. |
|---|---|---|---|---|
| *1927* | *gm.* | *gm.* | | |
| July 25–26 | 5 46 | 30.6 | 5 60 | 2 gm. phlorhizin. |
| "   26–27 | 3 87 | 18 6 | 4.81 | 1  "        "       shivered 5 hrs. |
| "   27* | 0 68 | 3.0 | 4 41 | |
| "   27–28 | 8 21 | 38 5 | 4 69 | 2 gm. phlorhizin, 16 gm. glucose. |
| "   28* | 0 90 | 3 9 | 4 33 | |
| "   28–29 | 7 22 | 40 8 | 5 65† | 2 gm. phlorhizin, 16 gm. glucose. |
| Subtotal | 16 33 | 83.2 | | |

Extra glucose excreted on basis D:N ratio 4.37‡: 1 = 11.8 gm. + 6.2 oxidized§ = 18.0 gm.

Extra glucose excreted on basis D:N ratio 3.65:1 = 23.6 gm. + 6.2 oxidized = 29.8 gm.

Glucose administered = 32 gm.

\* Catheterized specimen taken just before the administration of glucose.

† D:N ratio of 4.23 obtained for the night urine was used in the calculation of the respiratory data of July 29, Experiment 4.

‡ Average of the D:N ratios of 4.41 and 4.33 obtained just before the administrations of glucose.

§ The respiratory experiment on July 27, 1927, was unsatisfactory because of restlessness of the animal. Therefore, we assumed that the amount of glucose oxidized on that day was the average of the quantity found on July 28 and 29 (3.4 and 2.2 gm. respectively).

or roughly 30 per cent. As mentioned in the previous paragraph, the amount of carbohydrate calculated as oxidized would on this basis, for the average of the four experiments, be increased from 2.9 to 3.7 gm.; this is comparable to the variation found by Wierzuchowski. These two calculations represent the probable extremes and the true value would lie at some unknown intermediate point which, according to Brocklehurst and Henderson

TABLE VII.

*Urinary Data, Experiment 5.*

| Date. | Duration. | Total N. | Glucose. | D:N ratio. | Extra glucose. | | | Comments. |
|---|---|---|---|---|---|---|---|---|
| | | | | | On varying ratios. | On ratio 3.65:1. | On ratio 2.80:1. | |
| *1928* | *hrs.* | *gm.* | *gm.* | | *gm.* | *gm.* | *gm.* | |
| Sept. 8–9 | 23 75 | 5 90 | 28.5 | 4.83 | | | 7.0 | 12.0 | Last feeding 3.30 p.m., Sept. 5. 2 gm. phlorhizin, Sept. 8. 1 gm. phlorhizin, Sept. 9. |
| "  9–10 | 23 92 | 7 36* | 24.9* | 3.37 | | | 2 0 | 4.2 | 1 gm. phlorhizin, Sept. 10. |
| "  10 | 6 | 2 61* | 9 3* | 3 57 | | | 0 2 | 2 0 | 30 cc. Fisher's solution; no epinephrine. |
| | 6 08 | 2 37 | 10 1 | 4 24 | 1 83† | 1 4 | 3.4 | 30 cc. Fisher's solution; 1.2 cc. epinephrine (1:1000) intravenously. |
| Sept. 10–11 | 6 | 2 10 | 12 7 | 6.05 | 5.42† | 5 0 | 6.8 | 30 cc. Fisher's solution; 1.2 cc. epinephrine. |
| "  11 | 6 | 3 36 | 12 1 | 3 65 | 0 39† | 0.2 | 2 6 | 30 cc. Fisher's solution; 0.6 cc. epinephrine. |
| | 6 | 2 92 | 16 2 | 5 55 | 6 62‡ | 5 5 | 8 0 | 2 gm. phlorhizin; 16 gm. glucose; R.Q. indicates 7.14 gm. oxidized. |
| | 6 | 2.91§ | 8.5§ | 2 91 | | 2 2 | | |
| Sept. 11–12 | 12 | 6 08§ | 16 5§ | 2 72 | | 5 7 | | |
| Total ...................................... | | | | | 39.1 — (16.0 — 7.1) = 30.2 gm. glucose to be accounted for. | | | |

* Average D:N ratio 3.47.
† D:N ratio of 3.47 used.
‡ Average D:N ratio 3.28 used.
§ Average D:N ratio of 2.80.

(3) would be nearer the smaller value and according to Shaw (21) nearer the higher value.

Our experiments confirm those of Wierzuchowski and of Deuel, Wilson, and Milhorat by showing that a definite rise in the respiratory quotient follows the administration of glucose to completely phlorhizinized dogs; this rise, if influenced at all, is minimized by the simultaneous retention of carbon dioxide due to the decrease in the acidosis, as evidenced by a rising carbon dioxide-combining power. The amount of carbohydrate, then, actually metabolized may be somewhat greater than that indicated by the regular non-protein respiratory quotient.

Richardson (16) has recently made a complete review of the significance of the respiratory quotient. However, the temporary rise in respiratory quotient, so far as we have actual knowledge, may indicate either an increased rate of fat formation or an increased rate of carbohydrate combustion. Either of these interpretations implies an even, continuous acceleration of all the intermediate stages involved in either process. As yet students of general metabolism have only in the most superficial way considered the part that might be played by a temporary acceleration or retardation of some intermediate phase of the metabolic processes and in this respect have possibly paid too little attention to the mass of facts on this subject which have been brought out by Thunberg and his school. Ahlgren (1) in his Mayo Foundation lecture gave an excellent review of the present state of knowledge of this subject, with a suggestive correlation of the various facts known on the basis of Wieland's dehydrogenation theory of oxidation. According to this theory oxidation, for example of a substance like succinic acid, proceeds in a series of steps: (1) oxidation by dehydrogenation, (2) hydration or addition of water, and (3) splitting off of carbon dioxide or decarboxylation. A transient relative acceleration of the dehydrogenation stage would temporarily lower the respiratory quotient, whereas a transient relative acceleration of a decarboxylation stage would correspondingly raise the respiratory quotient and the hydration stage would leave it unaffected. Such a possibility is illustrated by the fact that when succinic acid, which has a respiratory quotient of 1.14, is added to the medium in experiments on tissue metabolism on fresh muscle hash, the respiratory quotient is definitely decreased,

indicating the formation of fumaric acid by dehydrogenation. The exact pathway by which carbohydrate is utilized is not known but that it proceeds through a series of steps is highly probable; it is also possible that one or another step may, by various procedures, be hastened or retarded temporarily more than another, thus producing corresponding temporary shifts in the respiratory quotient. In the building up of fat from carbohydrate intermediate stages must be passed through and it would seem unlikely, with varying quantities of the beginning and end-products, that the entire process should necessarily proceed at uniform speed. The mere fact that certain stages are apparently reversible implies a relative variation in the quantities of the intermediate stages as well in the end-products. If there is such a variation in speed of one set of reactions compared to another series, a slight temporary variation in the respiratory quotient would become manifest although the quantity of the intermediate product so retained would be too small to be revealed by our present methods of analysis. Variations in the respiratory quotient follow the ingestion of carbohydrate and other foods; also they can be temporarily influenced by such substances as insulin and epinephrine. Although as yet we have not been able to interpret such variations in terms of intermediate metabolism, it seems as though we are at the point where we may legitimately inquire whether or not a transient rise in the respiratory quotient may not possibly signify, at least in part, an increased rate of a decarboxylation stage (or a retardation of a dehydrogenation stage).

Cathcart and Markowitz (4) have recently questioned the validity of assuming that rises in the respiratory quotient over short periods which do not go above 1.00 necessarily indicate an accelerated rate of combustion and in this have voiced the doubts of all careful workers in the subject. Therefore, our calculations showing how much carbohydrate can be accounted for on the assumption of combustion must not be taken to indicate that we consider such a combustion is proved by such a temporary rise in the respiratory quotient; it is only a quantitative expression of one of the possibilities; the other alternatives cannot at present be expressed quantitatively. If the fact is borne in mind that for short periods other alternatives are possible, then there is considerable advantage in expressing one of the possibilities in a quantita-

tive manner. The converse must also be remembered; namely, that there is no real evidence that this rise in respiratory quotient does not indicate increased combustion of carbohydrate; on the whole the agreement found by Lusk under those conditions between direct and indirect calorimetry is sufficiently close to favor the latter interpretation although the accuracy of the apparatus is not quite sufficient to exclude the other possibilities.

A further fact which complicates the interpretation of the significance of variations in the respiratory quotient is the following: It is well known that by feeding carbohydrate the respiratory quotient will rise well above 1.00; it is quite easy to obtain in geese or pigs a respiratory quotient of 1.3 or more; it is possible even in man to obtain quotients of 1.1 after the administration of levulose. This is possible because the storage capacity for fat is nearly unlimited in hogs and geese and even large in man, especially when there is a tendency to obesity. The condition is much different in the case of an assumed formation of carbohydrate from fat as the storage of carbohydrate is in comparison very small and probably does not, in man, materially exceed 200 gm. Therefore, even if the carbohydrate stores are greatly depleted there is no possibility or necessity of an intense and long continued storage of carbohydrate formed from fat. Therefore, even if this reaction does take place, it would in all probability do so at a very slow rate and be hard to detect as it would only cause a slight lowering of the average respiratory quotient. The argument, therefore, against the formation of carbohydrate from fat, based on the fact that we do not find large depressions of the respiratory quotient below 0.7, corresponding to the marked elevation of the quotient above 1.0 occurring when fat is formed from carbohydrate, is after all not founded on a valid premise. Changes in the respiratory quotient by intermediate stages of metabolism are only produced by storage (or excretion) of the product supposed to be formed; since the possible stores of carbohydrate are very small the quantity retained could only affect the average quotient relatively little and only for a relatively short time. On the other hand, knowing the limitations of the method does not materially benefit the argument of those who insist that carbohydrate is formed from fat, especially as the foregoing would not hold if the sugar so formed was excreted; under these conditions

the quotient could be greatly depressed if large quantities of sugar were formed from fat and excreted.

In Tables IV to VII are shown the urine analyses for the respiratory experiments, Nos. 1, 2, 3, and 5; urine was not obtained for Experiment 4 since the condition of the dog was becoming unsuitable and it was considered inadvisable to keep it under phlorhizin for the completion of the 24 hour period. In Experiment 1 in which a small amount of urine was unfortunately lost, 10 gm. of extra glucose were excreted; the total amount accounted for, including that which was supposedly oxidized, amounted to 12.7 gm. or considerably less than the 16 gm. administered. In Experiment 2, 16.8 gm. of extra glucose were excreted and 3.1 gm. oxidized, making a total of 19.9 gm. accounted for, or 3.9 gm. more than the amount given. However, in the interval between this and the preceding experiment the dog was given in addition to the standard maintenance diet considerable quantities of carbohydrate in the form of Karo corn syrup. In Table VI the analysis of urine is recorded for 2 consecutive days on each of which 16 gm. of glucose were given; in this experiment the total amount of extra glucose in the urine, calculated on the average D:N ratio (4.33:1) of the catheterized specimens taken just before the administration of glucose, plus the quantity supposedly oxidized, amounts only to 18 gm., and if the ratio of 3.65:1 is used it amounts to 29.8 gm., in either case less than the 32 gm. administered. The ratio 4.33:1 suggests that there were still considerable stores of carbohydrate available; however, it is also well to refer to the observation of Sansum and Woodyatt (19, 20) who showed that phlorhizinized dogs, under the influence of a narcotic or after an injection of acetaldehyde, may have a temporary, but not necessarily parallel, retention of glucose, nitrogen, and acetone bodies. The nitrogen retention was sometimes relatively greater than that of glucose and hence caused a purely fictitious rise in the D:N ratio with an apparent excretion of extra glucose, whereas there might have been little, if any, increase in the total glucose excreted. Although the animals in our experiments were not under a narcotic, the possibility of slight variations in the relative rates of excretion of nitrogen and glucose over short periods must be borne in mind.

Furthermore, it is difficult to deplete the carbohydrate stores

to a constant minimal level, a fact repeatedly emphasized by Woodyatt (24) and by Lusk, and a definite standard and constant technique must be used. For many years it has been known that large quantities of glucose remain apparently unaccounted for when considerable amounts are injected into normal animals. The recent experiments of Folin, Trimble, and Newman (9) showing that the subcutaneous and some other tissues can act at least as a temporary reservoir for considerable quantities of glucose, while helping to clear up the fate of injected glucose at the same time introduce another variable into experiments which deal with the origin of extra glucose. It is possible that some such factor may, at least in part, account for the extra glucose which Chaikoff and Weber (5) obtained after previous heavy feeding with carbohydrate and which they believe originated from fat. With this in view it is interesting to note that in the experiments of Chaikoff and Weber the total amount of extra glucose eliminated during the entire experiment is much less than that found by Deuel, Wilson, and Milhorat who obtained from the phlorhizination of well fed dogs a grand total of 111 gm. of glucose from sources other than protein; of this, 54 gm. were eliminated by the kidneys, the rest being accounted for on the basis of the respiratory quotient by combustion or fat formation. In depancreatized animals, which just before the experiment had been especially well fed, Chaikoff and Weber found in a larger dog a total of 64 gm. of extra glucose in the urine; Macleod and Markowitz (14) obtained in one dog 84 gm. on the basis of a $D:N$ ratio of 2.8:1 and 50 gm. on the basis of a ratio of 3.65:1; in another small dog weighing only 4 kilos they calculated 74 gm. of extra glucose. It is doubtful whether these figures are sufficient to prove by themselves that the sugar excreted could not have come from carbohydrate stores well filled by high feeding, especially as the amount of sugar oxidized as indicated by the respiratory quotients after withdrawal of insulin in depancreatized dogs is not nearly as great as that found by Deuel, Wilson, and Milhorat in phlorhizinized animals; in fact, it is hardly probable that any is so utilized. Therefore, as the depancreatized dog would oxidize little if any of its carbohydrate store, there would remain a larger quantity to be eliminated by the kidney. The slight excess of extra glucose over and above that ingested, found by Deuel, Wilson, and Milhorat in

five of their eight experiments may be due to the fact that they did not reduce the carbohydrate reserves in their dogs to the minimal level. In fact, we feel convinced that this was not done in our Experiment 2 as in the interval between the experiments extra carbohydrate in the form of Karo corn syrup had been added to the standard diet, a procedure not done in the preliminary periods in the other experiments. This interpretation is confirmed by the experiments of Chambers and Coryllos (6) who did not find excess elimination of glucose whose origin could be traced to fat in depancreatized dogs that had been fasted for 48 hours before operation to render both the nitrogen excretion constant and to reduce the general carbohydrate stores to a minimal standard basal level.

It has been clearly shown by the studies of Ringer and by Sansum and Woodyatt that extra glucose can be obtained from the completely phlorhizinized dog by the use of epinephrine unless extreme precautions are taken to reduce the carbohydrate stores of the animal to a minimum. The results of these investigators demonstrate that repeated injections of epinephrine into the completely phlorhizinized dog will result in the excretion of less and less extra glucose and will finally fail to cause any elevation in the D:N ratio or in the absolute amount of glucose eliminated. When this stage has been reached the animals may be considered to have their carbohydrate stores reduced to the absolute minimum. Sansum and Woodyatt and Woodyatt have emphasized the marked difficulty in reducing carbohydrate stores to the minimal level and have shown that the animal tissues may contain only minimal quantities of glycogen with a D:N ratio of 3.65:1 or 2.80:1, neither ratio being in itself indicative of the degree of deglycogenation of the liver.

It has been suggested by Macleod (13) that the excess glucose which was occasionally obtained in the studies of Deuel, Wilson, and Milhorat is possibly to be explained by the conversion of fat into carbohydrate. The work of Chaikoff and Weber has been given the same interpretation. Our Experiment 5, the data for which are given in Table VII, does not supply evidence for such an assumption. The details are as follows: The animal was fasted for 2 days and then phlorhizinized as usual. On the 5th day of fasting and the 3rd day of phlorhizination the urine was

collected by catheter every 6 hours, and during the fourth, fifth, and sixth periods, the animal was given epinephrine (1:1000) intravenously. Extra glucose was obtained in the fourth and fifth periods but practically none in the sixth, this indicating that the carbohydrate reserves had been reduced to or nearly to the minimal level. Following the intravenous injection of 16 gm. of glucose the following amounts were accounted for: The extra glucose excreted in the urine amounted to 5.5 gm. on the basis of the D:N ratio of 3.65:1 found in the period just preceding; to this should be added the amount of glucose oxidized, on the basis of the rise in respiratory quotient, which amounts to 7.1 gm., making a total of 12.6 gm. or definitely less than the 16 gm. given. If the D:N ratio of 3.28:1 is used, which is the mean of the ratios in the immediately preceding and following periods, 13.8 gm. of glucose are accounted for, which is also less than the amount given.

In the 18 hours following the 6 hour period in which glucose was administered, the D:N ratio exactly corresponded to the Minkowski ratio of 2.80:1. As Macleod and his pupils have usually used the ratio of 2.80:1 in their calculations and also have assumed that the glycerol fraction of the fat molecule is excreted as glucose, we have made the following calculation of our data on the same basis. In the 95 hours following the beginning of phlorhizination the dog excreted in the urine a total of 39.1 gm. of extra glucose. From this should be subtracted the 16.0 gm. of glucose given intravenously, less the 7.1 gm. accounted for by the elevation of the respiratory quotient; the net amount of extra glucose to be accounted for, therefore, is 30.3 gm. From the data used by Chaikoff and Weber it can be assumed that 2 gm. of glucose could readily come from the liver of a dog weighing 15 kilos. To this can also be added the amount of glucose arising from the glycerol fraction of the fat; as the basal metabolism of the dog while on phlorhizin averaged more than 27 calories each hour, the total metabolism as the result of muscular movements must have averaged 10 per cent higher or about 30 calories each hour for 96 hours; therefore, the amount of fat burned would be approximately 300 gm. of which 10 per cent, or 30 gm., could be assumed to come from the glycerol fraction as glucose. Finally we must allow for the glucose disappearing from the blood and

tissue fluids in equilibrium with the blood; as the dog had previously only been on a maintenance diet and had in addition been fasted for 2 days before phlorhizin was started, we cannot assume that there was any great reserve in the tissues. A conservative estimate, therefore, of the blood and fluids in equilibrium with the blood is made at 20 per cent instead of a possible 60 per cent of the body weight or 3000 gm., and as the blood sugar after phlorhizination would drop approximately from 0.10 to 0.05 gm. we would obtain about 1.5 gm. The total amount of carbohydrate which can easily be accounted for without assuming that fat is converted to carbohydrate or the existence of any unknown reserves would amount, therefore, to more than 33 gm., which amount is considerably in excess of the 30.3 gm. of extra glucose actually excreted. By this method of calculation our data do not furnish evidence that any of the glucose excreted by the phlorhizinized dog is due to the conversion of fat to carbohydrate.

On the other hand, the work of Lusk and his pupils, ever since the original paper by Reilly, Nolan, and Lusk (15), has strengthened the evidence that in phlorhizinized dogs the D:N ratio is approximately 3.65:1 and that variations from this ratio are apparently due to changes in the amount of carbohydrate stored. Calculating the experiment on the 3.65 basis brings out very interesting facts, as shown in Table VII. During the 96 hours of the experiment 138.7 gm. of glucose were excreted and 16 gm. were administered, of which 7.1 gm. were apparently accounted for by the rise in respiratory quotient following its administration. The amount of glucose coming from protein and reserve stores would therefore be $138.7 - (16.0 - 7.1)$ or 129.8 gm., which divided by 35 6, the amount of nitrogen eliminated, gives a D:N ratio of 3.64:1. The stores of carbohydrate originally present at the time phlorhizination was begun were too small materially to affect the average ratio over such a long period because the dog had been fasted for 2 days before any phlorhizin was administered. These figures would indicate not only that glucose had not been formed from fat but that even the glycerol fraction of fat was not excreted as sugar. Lusk has found little evidence to indicate that the glycerol fraction of the fat molecule is eliminated as glucose in the phlorhizinized animal. The low D:N ratios of the seventh and eighth periods are probably due to the retention

of glucose following its administration after the excessive depletion produced by the epinephrine in the fourth and fifth periods. This retention suggests that excessive depletion of the carbohydrate stores by epinephrine is not as good a method of preparing dogs for experiments on the recovery of glucose as the standard fasting preparation usually used by Lusk. The variations in the D:N ratio in this experiment were balanced on account of its long duration but, as has been shown, essentially the same result in estimating the recovery and utilization of the ingested glucose is obtained if these variations are allowed for by taking the mean of the ratio of the period just preceding and following the glucose administration, according to the custom introduced by Lusk.

### CONCLUSIONS.

1. After several days of complete phlorhizination the basal metabolic rate in dogs is markedly elevated and may reach 90 per cent above the normal value.

2. The administration of glucose to the completely phlorhizined dog causes a definite decrease in the high level of heat production and a definite rise in the respiratory quotient. If the rise in the respiratory quotient is taken as indicating an increase in the oxidation of glucose, it is found that from 18 to 25 per cent of the ingested glucose is oxidized in from 5 to 7 hours.

3. In phlorhizinized dogs, when proper precautions are taken, the amount of glucose accounted for by oxidation and excretion in the urine will not exceed the amount given; therefore, the results from these experiments do not yield evidence in favor of the assumption that carbohydrate is formed from fat.

4. The significance of both the respiratory quotient and the D:N ratio is considered with special reference to possible errors in their interpretation.

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# SULFUR IN PROTEINS.

## IV. THE EFFECT OF ALKALIES UPON CYSTINE.*

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### INTRODUCTION.

In the first paper in this series Hoffman and Gortner[1] reviewed the literature dealing with the forms of sulfur which might be present in the protein molecule, including the effect of acids and alkalies upon the sulfur-containing amino acid, cystine. Accordingly no good purpose would be served by repeating their historical discussion.

Hoffman and Gortner showed that cystine is relatively stable to long boiling with 20 per cent hydrochloric acid, the only important change which is brought about by such treatment being the racemization of the *l*-cystine to an inactive form. They showed that the *i*-cystine so produced differs in certain physical and chemical properties from the normal *l*-cystine. In a later paper Gortner and Hoffman[2] report a comparative study of certain derivatives of *l*-cystine and their *i*-cystine, and show that the crystal form and physical properties of the derivatives are different in every instance. Attempts to resolve the *i*-cystine into optically active modifications were not successful, so that they were unable to decide whether or not the *i*-cystine represented a racemic mixture or perhaps a *meso* form of the amino acid.

Immediately following the study of the effect of acid upon cystine, studies were inaugurated to investigate the action of

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[1] Hoffman, W. F., and Gortner, R. A , *J. Am. Chem. Soc.*, **44**, 341 (1922).

[2] Gortner, R. A., and Hoffman, W. F , *J. Biol. Chem.*, **72**, 433 (1927).

alkalies upon cystine. The data included in the present paper were largely accumulated during the year 1924-25, but publication was withheld in the hope that certain of the organic compounds resulting from the alkaline decomposition of cystine could be isolated in pure form and identified. It has not been possible to complete this latter part of the problem. However, since one of us (S) can no longer continue the work, it has seemed advisable to present such phases of the problem as are complete.

<div align="center">EXPERIMENTAL.</div>

*Problem.* — In the earlier discussion of the literature dealing with the action of alkalies upon sulfur-containing proteins, it was pointed out that the early workers recognized two forms of sulfur, *i.e. loosely bound* and *firmly bound* sulfur. The loosely bound sulfur was differentiated from the firmly bound sulfur by the fact that when proteins were heated with 10 per cent sodium hydroxide solution in the presence of lead acetate, the loosely bound sulfur was converted into sulfide sulfur with the corresponding formation of lead sulfide. Varying amounts of loosely bound sulfur were reported by different workers for the same or for different proteins. Following the isolation of cystine, it was early noted that only a part of the cystine sulfur was removed as lead sulfide under the treatment noted above.

No thorough study of the effect of alkalies upon pure cystine had been reported in the literature prior to the time that the present investigations were undertaken. Andrews[3] has recently reported studies dealing with this problem. His contribution will be discussed after our data have been presented.

*Material.*—We have used in our study both the natural *l*-cystine isolated from human hair and a preparation of *i*-cystine prepared by boiling *l*-cystine with hydrochloric acid, following the general procedure already noted by Hoffman and Gortner.

The method used in preparing the *l*-cystine is that described by Gortner and Hoffman.[4] Approximately 300 gm. of this material were prepared. The preparation contained 11.66 per cent nitrogen, 26.84 per cent sulfur, and had an optical rotation of $[\alpha]_D^{20} =$

[3] Andrews, J. C., *J. Biol. Chem.*, **80**, 191 (1928).

[4] Gortner, R. A., and Hoffman, W. F., in Marvel, C. S., Organic syntheses, New York, **5**, 39–41 (1925).

−201.01°. Accordingly the preparation can be regarded as practically pure *l*-cystine.

In order to be sure that all of the *l*-cystine was converted into the inactive modification, 150 gm. of the *l*-cystine were boiled with 4 liters of constant boiling hydrochloric acid for 6 days. The *i*-cystine was isolated from this solution by distilling off the excess of hydrochloric acid *in vacuo*, dissolving the cystine hydrochloride in water, decolorizing the solution with norit, and precipitating the *i*-cystine by adding sodium acetate to the solution until the Congo red test for mineral acids was negative. The *i*-cystine was repurified by dissolving in a dilute solution of ammonium hydroxide and reprecipitating by the addition of acetic acid. The product so prepared agreed in all particulars with the preparation described by Hoffman and Gortner. It was entirely optically inactive and on analysis was found to contain 10.96 per cent nitrogen and 24.82 per cent sulfur, indicating a purity of 93.48 per cent. The impurity was almost wholly ash and moisture.

*Method.*—In studying the alkaline decomposition of cystine we have made the following determinations: (1) the nitrogen evolved as ammonia during the heating process, (2) the residual nitrogen remaining in the hydrolysate following the active boiling, (3) the nitrogen precipitated by phosphotungstic acid from the residual solution, (4) the sulfur evolved as hydrogen sulfide following an acidification of the residual alkaline solution, (5) the residual sulfur remaining in the hydrolysate after acidification, aeration to remove hydrogen sulfide, and filtration to remove elemental sulfur, and (6) the cystine in the residual hydrolysate as determined by the bromate titration method of Okuda:[5]

The apparatus which was used for this study is essentially the same as that shown in Fig. 1 of the previous study of Hoffman and Gortner[1] with the exception that a series of traps and Truog towers containing a known quantity of standard sulfuric acid was inserted in the train immediately following the reflux condenser, and immediately preceding the Truog towers containing the cadmium sulfate, which was used to collect the hydrogen sulfide.

An additional modification of the earlier technique lay in the fact that separate samples of cystine were used for each time

[5] Okuda, Y., *J. Coll. Agric., Imp. Univ. Tokyo*, **7**, 69 (1919).

interval instead of taking aliquots at various times from a larger sample. This necessiated replacing the large hydrolyzing flask of the earlier study with a 500 cc. flask in which was placed the solution of alkali which was used and to which was added the indicated amount of cystine. No attempt was made to carry out the study in the absence of atmospheric oxygen. As a matter of fact a slow current of air was aspirated through the apparatus during the entire period of boiling. The time noted in Tables I to IV is taken from the time active boiling began in the solution. Undoubtedly the results would have been more consistent, had the heating been carried out in an oil bath at constant temperature. Unavoidable differences in rate of heating and intensity of boiling probably account, at least in part, for certain of the discrepancies which will be noted in the following data.

The details of the analytical procedure differ in no important essential from those recorded in the earlier paper, and accordingly they will not be repeated here. Probably some of the apparent discrepancies in the tabular data are accounted for by the fact that many of the determinations were carried out on aliquots of the residual hydrolysate. Accordingly, the "experimental error" of an analysis is correspondingly magnified. Thus, the analytical figures for residual nitrogen and residual sulfur in the hydrolysate, as shown in Tables I and II, were obtained on aliquots of $\frac{1}{20}$ or $\frac{1}{10}$ of the total; those for hydrogen sulfide on $\frac{1}{4}$ of the total; those for "cystine by bromate method" on $\frac{1}{50}$ of the total and those for amino nitrogen on $\frac{1}{250}$ of the total.

<div align="center">EXPERIMENTAL.</div>

(a) *Nitrogen and Sulfur Changes.*—Tables I to IV present the experimental data on the decomposition of *l*- and *i*-cystine when boiled with 1 per cent and 5 per cent solutions of sodium carbonate; with 6.5 per cent solution of crystalline barium hydroxide; with 20 per cent solutions of sodium hydroxide and potassium hydroxide; with a 6 per cent solution of strontium hydroxide; with a 5 per cent suspension of calcium hydroxide; and with mixtures of sodium hydroxide and strontium hydroxide.

(b) *Attempts to Isolate Pure Organic Compounds from Cystine Decomposition Products.*—Three separate attempts have been made to identify the organic decomposition products which result when

| Alkaline solution used. | Material. | Weight of cystine taken | Time of boiling | N distribution in per cent of original N present in cystine | | | S distribution in per cent of original cystine S | | Cystine in residual hydrolysate (by bromate method). | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | N evolved as NH₃ | Residual N in hydrolysate | N precipitated by phosphotungstic acid | S evolved as H₂S | Residual S in hydrolysate | | |
| | | *gm.* | *hrs* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *gm.* | *per cent of original* |
| 250 cc. 1 per cent Na₂CO₃. | l-Cystine. | 2 50 | 2 | 3 43 | 95 54 | 69 81 | 0 26 | 92 57 | 2 271 | 90 8 |
| 250 " 1 " " " | " | 2 50 | 4 | 13 60 | 88 50 | 47 34 | 1 45 | 81 35 | 2 082 | 83 3 |
| 250 " 1 " " " | " | 2 50 | 12 | 32 40 | 67 06 | 13 89 | 6 32 | 66 97 | 1 619 | 64 7 |
| 250 " 1 " " " | " | 2 50 | 24 | 44 47 | 57 29 | 3 77 | 4 79 | 55 54 | 1 244 | 49 8 |
| 250 cc. 1 per cent Na₂CO₃. | i-Cystine. | 2 3375* | 2 | 4 91 | 94 89 | 56 20 | 0 76 | 54 12† | 2 217 | 94 8 |
| 250 " 1 " " " | " | 2 3375* | 4 | 7 86 | 92 34 | 44 53 | | 52 34† | 1 884 | 80 6 |
| 250 " 1 " " " | " | 2 3375* | 12 | 37 54 | 63 69 | 13 69 | 6.77 | 30 09† | 1 307 | 55 9 |
| 250 " 1 " " " | " | 2 3375* | 24 | 43 81 | 57.11 | 10 40 | 4 51 | 19 92† | 1 154 | 49 4 |
| 250 cc. 5 per cent Na₂CO₃. | l-Cystine. | 2 50 | 2 | 6 40 | 89 53 | 62.09 | 0 65 | 89 90 | 2 217 | 88 7 |
| 250 " 5 " " " | " | 2 50 | 4 | 15 85 | 81 30 | 45 89 | 3 99 | 83 55 | 1 910 | 78 4 |
| 250 " 5 " " " | " | 2 50 | 12 | 33 02 | 66 69 | 28 54 | 15 22 | 66 14 | 1 424 | 57 0 |
| 250 " 5 " " " | " | 2 50 | 24 | 42 42 | 56 60 | 18 18 | 16 89 | 58 82 | 1 180 | 47 2 |
| 250 cc. 5 per cent Na₂CO₃. | i-Cystine. | 2 3375* | 2 | 8.83 | 87.23 | 33 94 | 0 85 | 100 8 | 2 206 | 94 3 |
| 250 " 5 " " " | " | 2 3375* | 4 | 19 85 | 77 55 | 22.66 | 4 91 | 89 85 | 1 875 | 80 2 |
| 250 " 5 " " " | " | 2 3375* | 12 | 25.09 | 68.61 | 19 34 | 20 85 | 66 83 | 1 433 | 61 3 |
| 250 " 5 " " " | " | 2 3375* | 24 | 45.64 | 55 29 | 8 94 | 18 37 | 56 92 | 1 226 | 52.4 |

* This weight represents 2.50 gm. of 93.48 per cent purity.

† For some unknown reason these values are too low. Possibly the wrong pipette was used for taking the aliquots. The values are, however, included, although they should not be considered in making comparisons.

cystine is boiled for an extended period of time with barium hydroxide.

In each instance 50 gm. of *l*-cystine were boiled for 24 hours with 4 liters of 6.5 per cent barium hydroxide.   The solution rapidly became colored light yellow after boiling began; this color deepening until at the end of 24 hours the hydrolysate was a deep yellow

TABLE II.

*Decomposition of l-Cystine and i-Cystine When Boiled for Varying Lengths of Time with 250 Cc. of 6.5 Per Cent Ba(OH)₂·8 H₂O.*

| Material | Weight of cystine taken | Time of boiling | N distribution in per cent of original N present in cystine | | | S distribution in per cent of original cystine S | | Cystine in residual hydrolysate (by bromate method ) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | N evolved as NH₃ | Residual N in hydrolysate | Amino N in hydrolysate | S evolved as H₂S | Residual S in hydrolysate | | |
| | *gm* | *hrs* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *gm* | *per cent of original* |
| *l*-Cystine. | 2 50 | 1 | 52 85 | 53 96 | 49 40 | 38 58 | 41 65 | 1 06 | 42 4 |
| " | 2 50 | 1 | 53 58 | 53 85 | 45 62 | 26 57 | | | |
| " | 2 50 | 2 | 64 04 | 38 45 | 37 05 | 52 64 | 44 87 | 0 93 | 37 2 |
| " | 2 50 | 2 | 59 13 | 46 38 | 50 56 | 49 87 | 34 46 | | |
| " | 2 50 | 2 | 61 84 | | | 60 01 | | | |
| ' | 2 50 | 5 | 68 77 | | 31 49 | 57 92 | 44 12 | 0 74 | 29 6 |
| " | 2 50 | 5 | 71 84 | 17 77 | 28 81 | 56 23 | 27 72 | | |
| " | 2 50 | 5 | 87 01 | | | 54 92 | | | |
| " | 2 50 | 24 | 79 91 | 26 75 | 22 73 | 59 28 | 20 52 | 0 40 | 16 0 |
| " | 2 50 | 24 | 81 56 | 10 53 | 20 93 | 56 03 | 16 03 | | |
| *i*-Cystine. | 2 3375* | 1 | 59 16 | 41 28 | 40 14 | 34 21 | 39 16 | 0 94 | 40 2 |
| " | 2 3375* | 2 | 59 40 | 29 89 | 31 39 | 54 22 | 33 84 | 0.84 | 35.9 |
| " | 2 3375* | 5 | 63 50 | 25 55 | 30 29 | 48 87 | 27 07 | 0 65 | 27.8 |
| " | 2 3375* | 24 | 80 95 | 11 57 | 15 32 | 73 27 | 14 50 | 0 34 | 14 5 |

* This weight represents 2.50 gm. of 93.48 per cent purity.

or orange.   A light yellow precipitate was formed, apparently consisting largely of elemental sulfur, inasmuch as the greater part of the precipitate was soluble in carbon disulfide and this solution on evaporation deposited the characteristic sulfur crystals.

Following the 24 hour boiling the solution was cooled; acidified

TABLE III.

*Effect of Various Solutions of Alkalies on Decomposition of l-Cystine as Measured by Changes in Nitrogen Distribution.* *

| Alkaline solution used | Weight of cystine taken | Time of boiling | N distribution in per cent of original N present in cystine | | |
|---|---|---|---|---|---|
| | | | N evolved as $NH_3$. | Residual N in hydrolysate | Amino N in hydrolysate |
| | gm | hrs | per cent | per cent | per cent |
| 60 cc  20 per cent NaOH | 0 50 | 2 | 7 89 | 92 70 | 75 73(?) |
| 30  " 20  "    "    " | 0 25 | 2 | 8 75 | 89 28 | 90 66 |
| 60  " 20  "    "    " | 0 50 | 4 | 12 18 | 92 72 | 92 95 |
| 60  " 20  "    "    " | 0 50 | 12 | 10 98 | 89 09 | 77 84 |
| 30  " 20  "    "    " | 0 25 | 24 | 12 39 | 86 88 | 83 34 |
| 200 cc  20 per cent KOH. | 4 00 | 6 | 1 87 | 95 01 | 96 88 |
| 60  " 20  "    "    " | 0 50 | 6 | 1 80 | | |
| 50 cc  5 5 per cent Ba(OH)$_2$ 8H$_2$O | 0 25 | 2 | 42 06 | 56 66 | |
| 50  "  5 5  "    "    " | 0 25 | 12 | 52 88 | 47 04 | |
| 50  " 12 0  "    "    " | 0 25 | 24 | 70 39 | | |
| 100 cc  5 per cent Ca(OH)$_2$ | 0 25 | 4 | 55 63 | | 45 41 |
| 100  " 5  "    "    " | 0 25 | 12 | 92 46 | 9 28 | |
| 50 cc.  6 per cent Sr(OH)$_2$·8H$_2$O. | 0 25 | 24 | 64 56 | 34 30 | |
| 30  " 20  "    " NaOH + 3 gm Sr(OH)$_2$ 8H$_2$O | 0 25 | 5 | 23 00 | | |
| 30 cc. 20  per  cent  NaOH + 3  gm Sr(OH)$_2$ 8H$_2$O. | 0 25 | 5 | 23 86 | | |
| (A) 50 cc. 5 per cent Na$_2$CO$_3$. | 0 25 | 2 | 10 30 | | |
| (B) Residue from (A). | | 5 | 14 42 | | |
| (C)    "        " (B). | | 12 | 24.72 | | |
| (D)    "        " (C). | | 7 | 2 74 | | |
| Total (A) — (D). | | 26 | 52 18 | | |

* In obtaining the ammonia nitrogen values of this table, use was made of the apparatus as devised by Holm, G. E., (*J. Am. Chem. Soc.*, **42**, 611–612 (1920)) to measure the ammonia formed by the alkaline decomposition of arginine. *Cf.* also Morrow, C. A., Biochemical laboratory methods, New York, 167 (1927).

with sulfuric acid, the barium sulfate was removed by filtration, the excess sulfuric acid was removed by adding an excess of washed barium carbonate, and barium was quantitatively determined in an aliquot of the resulting filtrate.   Standard sulfuric acid was then added to remove quantitatively the final traces of barium, and gave a barium- and sulfate-free solution.   This solution was evaporated to dryness *in vacuo,* and became a gummy mass, acid in reaction.

TABLE IV.

*Change of Optical Activity with Time during Boiling of l-Cystine with Barium Hydroxide and Sodium Carbonate Solutions.* *

| Alkali used. | Time of boiling. | Specific optical rotation of cystine in residual hydrolysate. |
|---|---|---|
| | *hrs* | *degrees* |
| Check. | 0 | −201 .01 |
| 6.5 per cent Ba(OH)₂·8H₂O. | 1 | −138 63 |
| 6.5  "   "   " | 2, 5, 24 | 0.0 |
| | | |
| 1 per cent Na₂CO₃. | 2 | −166 35 |
| 1  "   "   " | 4 | −128 63 |
| 1  "   "   " | 24 | −55 45 |
| | | |
| 5 per cent Na₂CO₃. | 2 | −166 35 |
| 5  "   "   " | 4 | −117 83 |
| 5  "   "   " | 12 | −86 64 |
| 5  "   "   " | 24 | −34 66 |

* The specific optical rotations were calculated from the polarimeter readings on the basis of the cystine originally added at the beginning of the experiment.

This gummy mass could be fractionated into three portions: (*a*) a portion soluble in ethyl ether (*b*) a portion insoluble in ether but soluble in absolute alcohol, and (*c*) a residue insoluble in either ethyl ether or alcohol but so soluble in water that it will absorb moisture from the air and pass to a gummy mass.

The ether-soluble portion from 50 gm. of cystine amounted to approximately 2.15 gm.   On evaporating to dryness and allowing to stand over sulfuric acid in a desiccator for several weeks, it solidifies to a glass.   It is only partially soluble in water, but completely soluble in dilute hydrochloric acid.   It gives a very

strong test for loosely bound sulfur when heated with lead acetate in the presence of alkali. The analyses for total nitrogen and for total sulfur showed that the extract contained 19.48 mg. of nitrogen and 127.63 mg. of sulfur, giving a nitrogen:sulfur ratio of 1:6.55. As yet no pure compound has been isolated from this fraction.

The alcohol-soluble fraction yields a dark red solution, and on evaporating off the alcohol a soft, sticky, red gum results. This on standing for several months solidifies to a glass. The alcoholic solution contains considerable amounts of loosely bound sulfur, as indicated by the characteristic formation of lead sulfide when warmed with lead acetate in alkaline solution. Analyses of this fraction for nitrogen and sulfur showed that 0.5096 gm. of nitrogen and 0.1834 gm. of sulfur were present, the nitrogen:sulfur ratio being 1:0.36.

Various attempts have been made to isolate pure compounds from this fraction. No volatile organic acids are present. In one series of experiments we obtained a colorless crystalline solid, m.p. 262–262.5° (uncorrected) and weighing 0.7 gm., and a second solid more or less similar in appearance to the first, m.p. 246–247° (uncorrected) and weighing 0.3 gm. We have not felt justified in attempting to determine the structure of these compounds with the amounts which are available but plan to study them in greater detail after preparing larger quantities.

The portion insoluble in absolute alcohol amounted in each instance to approximately 4.0 gm. If any undecomposed cystine remains following the alkaline hydrolysis, it should appear in this fraction. Accordingly we have devoted most of our attention to a study of this fraction. As already noted, this fraction is extremely hygroscopic, passing to a gummy mass on exposure to the moisture of the laboratory air. No means of purifying this compound have as yet been found aside from exhaustive extraction by absolute alcohol. The product is insoluble in nearly all organic solvents and so extremely soluble in water as to preclude the possibility of recrystallization. In one experiment 0.14 gm. of colorless prisms was obtained from a water-acetic acid-alcohol mixture. These crystals darkened at about 245°, but did not melt below 295°. In other experiments it was not possible to reproduce this crystal formation.

The alcohol-insoluble fraction contained 7.53 per cent nitrogen (7.61 per cent of amino nitrogen by Van Slyke's method) and 20.29 per cent sulfur on a moisture- and ash-free basis, giving a ratio of nitrogen : sulfur of 1:2.69.   The nitrogen : sulfur ratio of pure cystine is 1:2.29.   Accordingly, in this fraction we have a nitrogen . sulfur ratio very similar to the nitrogen : sulfur ratio of the original cystine.   This fraction likewise contains appreciable quantities of sulfur which are split off to form lead sulfide when boiled with an alkaline solution of lead acetate.   Attempts to form derivatives have yielded a small quantity of a phenyl-isocyanate, crystallizing in needles, melting after recrystallization at 173–173.5° (corrected) and containing 10.71 per cent nitrogen and 14.38 per cent sulfur.   Cystine phenylisocyanate should contain 11.72 per cent nitrogen and 13.39 per cent sulfur.   The isocyanate, therefore, approximates the composition of cystine isocyanate.   The melting point is somewhat lower than Gortner and Hoffman found for $l$-cystine phenylisocyanate, 181° (uncorrected).   The exact identification of this compound must await the preparation of larger quantities.

Our experience in working with the alcohol-soluble fraction leads us to suggest that in this fraction we are dealing with another compound, possibly isomeric in its chemical composition with cystine.   The physical properties, however, particularly the very high solubility in water, bear no relation to the physical properties of cystine.   To solve the problem of the nature of the compound or compounds present in this fraction will require very much larger quantities of material than we have worked with.

The above report is inserted at this point merely to indicate the complexity of the decomposition products and as a report of progress.   We are planning to work up approximately a kilo of cystine so as to obtain sufficient material to study the fractions noted above.   We had hoped to be able to isolate serine as one of the decomposition products.   From the study of the nitrogen and sulfur changes it appears highly probable that we may be able to isolate serine from the residual hydrolysate obtained by boiling with sodium or potassium hydroxide, inasmuch as these alkalies appear to remove the sulfur portion of the cystine molecule without appreciably affecting the amino group.   The difficulty in studying the organic decomposition products when sodium or potassium

hydroxide is used lies in the presence of the large amounts of water-soluble salts formed by the neutralization of the alkaline solution.

As already indicated, Andrews has made some studies of the alkaline decomposition of cystine. He allowed solutions of cystine to stand at room temperature in contact with 4 M sodium hydroxide and with 4 M sodium hydroxide to which lead acetate had been added. After standing 14 days only 8.3 per cent of the cystine sulfur could be precipitated as lead sulfide from the solution in which only sodium hydroxide was present. When, however, lead acetate had been present in the sodium hydroxide solution throughout the entire 14 day period, from 76.9 to 78.9 per cent of the cystine sulfur was precipitated as lead sulfide. He accordingly suggests that, "a high yield of lead sulfide requires the presence of plumbite during the whole time of decompositon of the cystine," and suggests that, "this supports the transitory character of the sulfide ion." He further notes that the concentration of the alkali plays a very minor part in comparison with the effect of the presence or absence of lead.

Andrews likewise studied the production of ammonia in the sodium hydroxide solutions, both with and without the presence of lead, and notes that "the effect of the lead was to more than double the rate of ammonia production."

In a study designed to elucidate the factors involved in. the action of the lead, he notes that, "the action is probably not catalytic in nature," that copper (introduced as copper sulfate) showed a much less marked effect than lead, that metallic copper had no effect, and that when ferric chloride was added, the iron had but little effect. Andrews concludes, "this very marked effect of lead is of highly specific nature," and even suggests that, "this specificity of lead in cystine chemistry leads to interesting speculations as to any possible connection with its very specific properties as a poison."

It will be noted from our Tables I to IV, inclusive, that the decomposition of cystine in solutions of sodium carbonate, barium hydroxide, and strontium hydroxide is as great or greater than Andrews found when lead was present in sodium hydroxide solutions, so that apparently *lead is not specific* in producing these

peculiar effects.   No certain explanation appears to be available for the anomalous behavior of the strong sodium and potassium hydroxide solutions.   As a matter of fact the sodium and potassium hydroxide series were run only after the paper by Plimmer and Lowndes[6] had appeared.   They note that boiling cystine for 6 hours with 20 per cent sodium hydroxide causes cystine to lose only 20 per cent of its nitrogen as ammonia.   Van Slyke[7] had previously reported a maximum of 18 per cent of cystine nitrogen as ammonia under similar conditions.   We had, prior to 1927, completed the data which are shown in Tables I and II and had used barium hydroxide in our studies, acting on the belief that barium hydroxide would produce less drastic decomposition than would the stronger sodium hydroxide.   In order to check the observations noted by Plimmer and Lowndes, the work reported in Table III was undertaken.   It will be seen that their findings are confirmed.   It will also be noted that potassium hydroxide of equivalent concentration causes appreciably less decomposition of cystine, in so far as the nitrogen is concerned, than does sodium hydroxide.

The anomalous effects, in so far as deamination is concerned, can hardly be due to hydroxyl ion concentration, for it will be noted that 1 per cent sodium carbonate produces approximately as much deamination as does 5 per cent sodium carbonate and nearly as much deamination as does the stronger barium hydroxide solution.   Calcium hydroxide produces somewhat more deamination than do any of the other alkalies tested.   Strontium hydroxide, when added to a solution of sodium hydroxide, increases deamination to a point intermediate between what would have been produced in the presence of strontium hydroxide alone or in the presence of the sodium hydroxide alone.   According to Andrews' work, lead hydroxide would be equally efficient in producing deamination and sulfur changes.

Turning now to the effect of alkalies upon the sulfur portion of the cystine molecule, it will be noted that boiling with 1 per cent sodium carbonate solution induces the formation of only relatively small amounts of sulfide sulfur, the main bulk of the sulfur remaining as organically combined sulfur in the residual hydrolysate.

[6] Plimmer, R. H. A., and Lowndes, J., *Biochem. J.*, **21**, 247 (1927).

[7] Van Slyke, D. D., *J. Biol. Chem*, **10**, 15 (1911–12).

5 per cent sodium carbonate produces appreciably more sulfide sulfur, reaching a maximum of 17 or 18 per cent after a 24 hour boiling in the alkaline solution. However, even in this case somewhat more than half of the sulfur remains in the form of organic sulfur in the residual hydrolysate. Boiling with barium hydroxide causes a much larger proportion of the sulfur to be removed from the cystine molecule. The great bulk of the sulfur so removed is later recoverable as hydrogen sulfide. In this series of experiments the residual sulfur and the sulfur evolved as hydrogen sulfide do not in most cases add up to 100 per cent. The discrepancy is due to the fact that colloidal sulfur is formed, possibly by the oxidation of sulfides, possibly as a primary product of the action of the alkali upon cystine. This elemental sulfur is lost when the acidified solution is filtered prior to the determination of the residual sulfur in the hydrolysate, and accordingly any elemental sulfur which is formed is unaccounted for in our scheme of analysis.

There seems to be an intimate relationship between the deamination mechanism and the sulfur changes which are taking place. We have every reason to believe that the strong sodium and potassium hydroxide solutions cause a rapid and complete loss of sulfur from the cystine molecule. The only concrete evidence that we have for this belief lies in the fact that the residual hydrolysates from the sodium hydroxide-cystine systems after acidification show little or no lead sulfide formation when they are subsequently boiled with alkaline lead acetate solutions, whereas all of the residual hydrolysates, even those from a 24 hour barium hydroxide boiling, still contain lead-blackening organic sulfur in appreciable amounts. Possibly deamination of cystine in an alkaline solution may be associated with an oxidation-reduction mechanism, such as is characteristic of cystine itself and that when that oxidation-reduction mechanism is inhibited by the elimination of sulfur from the molecule, then the $\alpha$-amino group becomes relatively stable. We hope to test out certain of these hypotheses in the near future, but some similar mechanism must be involved to account for the results which our data indicate.

The amino nitrogen determinations show that all of the nitrogen in the residual hydrolysate is still present in the form of free amino groups and that no intermolecular condensations involving the

α-amino group have taken place.   The residual nitrogen in the hydrolysate, the amino nitrogen in the hydrolysate, and the residual sulfur in the hydrolysate point rather definitely toward the conclusion that *cystine or some organic compound having essentially the same sulfur : nitrogen ratio as cystine is still persisting even after 24 hours of boiling with 6.5 per cent barium hydroxide.* Our data indicate that such compounds, amounting to approximately 16 per cent of the original cystine, are present at the end of 24 hours of boiling with the alkaline solution.   These values are further checked by the cystine as determined by Okuda's bromate method.   The bromate titrations show that when cystine is boiled with 6.5 per cent barium hydroxide solution, approximately 60 per cent of the cystine is decomposed during the 1st hour of boiling, approximately 63 per cent during 2 hours of boiling, approximately 70 per cent during 5 hours of boiling, and approximately 84 per cent during 24 hours of boiling.   It is difficult to picture a simple mechanism which will destroy 60 per cent during the 1st hour of boiling and only 84 per cent during 24 hours of boiling.

### SUMMARY.

Cystine has been boiled with solutions of the various alkalies for various lengths of time, and changes in the nitrogen and sulfur portions of the cystine molecule have been followed as an index of the rate and extent of decomposition of the cystine molecule. Attempts have also been made to identify specific organic compounds resulting from the action of barium hydroxide solutions upon cystine.

The data warrant the following conclusions:

1. Comparative studies of *l*-cystine and *i*-cystine boiled with alkaline solutions show that these two forms are characterized by the same rate of decomposition and the same extent of decomposition.

2. Boiling cystine with a 1 per cent solution of sodium carbonate causes very appreciable decomposition of the cystine molecule, the extent of decomposition increasing with time until at the end of 24 hours approximately half of the cystine has been decomposed.

3. Increasing the strength of the sodium carbonate solution to 5 per cent does not greatly increase the rate or extent of deamina-

tion or, apparently, of cystine destruction, although appreciably larger quantities of the cystine sulfur are converted into sulfide sulfur.

4. Boiling cystine with a 6.5 per cent solution of $Ba(OH)_2 \cdot 8 H_2O$ produces rapid deamination and loss of sulfur from the cystine molecule. Approximately 50 per cent of the cystine is destroyed in the 1st hour of boiling, the nitrogen being liberated as ammonia and the sulfur largely as sulfide sulfur. However, at the end of 24 hours of boiling there still remains approximately 15 per cent of organically bound amino nitrogen and an equivalent amount of organically bound sulfur.

5. Sodium hydroxide and potassium hydroxide solutions in 20 per cent concentration cause relatively slight deamination; after 24 hours of boiling approximately 83 per cent of the original nitrogen is still present as $\alpha$-amino nitrogen. There is some evidence that these solutions of strong alkalies cause rapid and complete removal of the sulfur from the cystine molecule.

6. A 6 per cent solution of strontium hydroxide and a 5 per cent suspension of calcium hydroxide bring about rapid deamination of cystine, the deamination produced by boiling cystine with a 5 per cent suspension of calcium hydroxide reaching 92 per cent during 12 hours of boiling.

7. The rate and extent of deamination does not appear to be a function of the hydroxyl ion concentration of the alkaline solution.

8. There appears to be an intimate relationship between the deamination mechanism and the rate at which sulfur is removed from the cystine molecule. It is suggested that deamination of cystine in alkaline solution may be associated with an oxidation-reduction mechanism and that when all, or nearly all, of the sulfur has been removed from the cystine molecule, then the $\alpha$-amino groups which still remain become relatively stable.

9. Contrary to the suggestions of Andrews, lead in sodium hydroxide solution is not specific in inducing extensive sulfur changes and deamination in the cystine molecule. Other weak alkalies, such as barium, strontium, and calcium hydroxide, are equally efficient.

10. Attempts to isolate pure organic compounds from the decomposition products resulting when cystine is boiled with 6.5 per cent barium hydroxide solution have shown that a complexity of compounds is present.

11. "Loosely-bound" organic sulfur is still present in such compounds following boiling of cystine with barium hydroxide for 24 hours.

12. Cystine, or some organic compound having essentially the same nitrogen: sulfur ratio as cystine, still persists in the solution after 24 hours of boiling of cystine with 6.5 per cent barium hydroxide.

13. It is suggested that this "compound" may be another "isomeric" form of cystine, differentiated from cystine by the fact that it is extremely soluble in water, so soluble in fact as to be hygroscopic.

14. The study of the organic chemistry of the decomposition products of cystine in alkalies is being continued.

# COMPOSITION OF BONE.

## VII. EQUILIBRATION OF SERUM SOLUTIONS WITH DICALCIUM PHOSPHATE.*

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It is generally taken for granted that bone consists chiefly of $Ca_3(PO_4)_2$ and that when calcium phosphate is deposited in the bones it is in the form of this compound, tricalcium phosphate. There have been several notable attempts to account for the deposition of tricalcium phosphate on the basis of solubility product considerations. Holt, La Mer, and Chown (1) interpreted their experiments as showing that "serum is normally supersaturated with tertiary calcium phosphate to the extent of more than 200 per cent." According to Holt (2) not only is normal serum supersaturated but also "Even in active rickets this ion product is greater than that required to precipitate $Ca_3(PO_4)_2$." Sendroy and Hastings (3) performed equilibration experiments with solid $Ca_3(PO_4)_2$; they concluded that supersaturation cannot be the sole explanation "for the apparently abnormal amounts of calcium in serum," and that the data indicate "that calcium exists in serum in abnormal amounts bound to some substance or substances which hold it in solution in unionized form."

In a previous communication (4) we presented evidence which indicates that serum does not contain abnormal amounts of calcium and that serum and inorganic serum solutions are not supersaturated but are undersaturated. Furthermore, the important substance appears to be $CaHPO_4$ and not $Ca_3(PO_4)_2$. Our

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* Part of the data contained in this communication was presented before the XIIIth International Physiological Congress at Boston, August 22, 1929, in a paper entitled, "Serum Calcium; Unsaturation *versus* Supersaturation."

calculations showed that in inorganic serum solutions with values of the empirical Ca × P product less than 30, the values of the ion product $[Ca^{++}] \times [HPO_4^=]$ are less than the solubility product of $CaHPO_4$, and that in solutions with Ca × P products ranging from 40 to 60, the values of the ion product $[Ca^{++}] \times [HPO_4^=]$ are very nearly equal to the solubility product of $CaHPO_4$.

The value for $K'_{s.p.}CaHPO_4$, the solubility product of $CaHPO_4$, was obtained by extrapolation from data in the literature.   The present paper gives the values of $K'_{s.p.}CaHPO_4$ which we obtained experimentally at the ionic strength of serum.[1]

### Methods and Materials.

Crystalline $CaHPO_4$ was made by precipitation from solutions whose pH was not greater than 5.5.   An approximately 0.1 M solution of $KH_2PO_4$ was mixed with a $CaCl_2$ solution of the same strength and pH.   Then very dilute $NH_4OH$ was added slowly, with stirring.   The precipitate contained no amorphous material. The crystalline $CaHPO_4$ was washed thoroughly and dried at 110°.   Gravimetric analysis gave a Ca:P ratio of 1.289.   This is in excellent agreement with the theoretical value of 1.291.

Inorganic serum solutions were made with sodium, potassium, magnesium, chloride, and $CO_2$* present in the same concentrations as in normal blood serum.   The variable constituents were calcium, phosphorus, and hydrogen ion.   The concentration of phosphorus was varied from 0.0 to 10.0 mg. per 100 cc.   The concentration of calcium also was varied from 0.0 to 10.0 mg. per 100 cc.   Such concentrations of calcium and phosphorus were employed as to vary the Ca × P product† from 0 to 65.   The ionic strength, $\mu$, of these inorganic serum solutions was approximately 0.14.

Calcium in the solutions was determined by the Kramer-Tisdall method; phosphorus was determined by the Fiske-Subbarow (7) method.   The pH determinations were made colorimetrically, using M/15 phosphate buffer solutions as comparison standards.

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[1] A preliminary report of these experiments was published in *Science* (5).

* A few solutions did not contain $CO_2$; these are indicated in the tables.

† This is the empirical product of Howland and Kramer (6) in which the Ca and P are expressed in mg per 100 cc.

*Calculations.*

As stated in a previous communication (4) we have set $[Ca^{++}]$ equal to the total calcium concentration obtained by analysis. The value of $[HPO_4^=]$ was obtained by means of the expression

$$[HPO_4^=] = \frac{[P]}{1 + 10^{(pK_2' - pH)}}$$

in which $[P]$ is the total concentration of phosphate obtained by analysis expressed in mols per liter; $pK_2'$ was calculated from the expression given by Sendroy and Hastings.

$$pK_2' = 7.15 - 1\,25\,\sqrt{\mu}$$

Jowett and Millet (8) have very recently reported a study of the ionization constants of phosphoric acid. At 37.5° they give the value 7.06 for $pK_2$ as compared with the value 7.15 given by Sendroy and Hastings. The latter investigators made their determinations at 20° and applied a temperature coefficient in order to obtain $pK_2$ at 38°. Jowett and Millet made their determinations of $pK_2'$ at 37.5° at one ionic strength and calculated $pK_2$ by means of Cohn's (9) relation

$$pK_2 = pK_2' + \frac{1\,5\,\sqrt{\mu}}{1 + 1\,5\,\sqrt{\mu}}$$

Jowett and Millet believe their value for $pK_2$ to be consistent with that deduced by Cohn for they state "$pK_2$ is calculated to be 7.127 at 25° and 7.058 at 37.5°. The value at 25° is close to the value 7.130 obtained by Cohn at 18°." In Cohn's paper the value for $pK_2$ is given as 7.16, based on the Sørensen value of the 0.1 N calomel electrode; Jowett and Millet stated, "Reduced to the G. N. Lewis standard for the hydrogen electrode, this value is 7.130." However, in a personal communication Cohn has informed us that if the Lewis, Brighton, and Sebastian value of the 0.1 N calomel electrode is employed, $pK_2$ becomes 7.19. The value which Sendroy and Hastings obtained for $pK_2$ at 20° was 7.18; there is thus good agreement between the value obtained by Cohn at 18° and that reported subsequently by Sendroy and Hastings for 20°. Apparently further

work is necessary to determine the exact value of $pK_2$ at body temperature.    Until this is done, we shall continue to use the value given by Sendroy and Hastings so as to conform with our earlier calculations.

### Preliminary Experiments.

In the experiments of Bassett (10) in which he concluded that $Ca_3(PO_4)_2$ and hydroxyapatite, $Ca_3(PO_4)_2 \cdot Ca(OH)_2$, were the solid phases at equilibrium, equilibration was carried on for weeks and months, and in some instances for over a year.    But he concluded that in spite of the protracted equilibration, equilibrium had not been attained in many cases.    He stated "In the case of the experiments at 25°, it has been found that, even in the most favorable circumstances, at least eighteen months are required for equilibrium to be reached."    Holt, La Mer, and Chown (1) equilibrated solutions continuously for 10 days and obtained paradoxical results.    Sendroy and Hastings equilibrated solutions from 20 hours to 8 days with solid $Ca_3(PO_4)_2$, and also obtained peculiar results.

In the experiments reported here, it was found that equilibrium is reached rapidly when $CaHPO_4$ is used as the solid phase.

About 20 cc. of distilled water were put into each of four test-tubes and 100 mg. of the crystalline $CaHPO_4$ were added to each. The test-tubes were tightly stoppered and shaken continuously in a machine.    One tube was withdrawn at the end of each hour. The pH was determined immediately after removal from the shaking machine.    The supernatant solutions after separation from the solid phase were analyzed for calcium and phosphorus. The results are given in Table I.    It is seen that equilibrium was attained after shaking for only 1 hour.

A similar set of experiments was next performed to see how long it takes to attain equilibrium when a serum solution is used. An inorganic serum solution was made up containing 8.0 mg. per cent of calcium and 3.0 mg. per cent of phosphorus.    This solution was analyzed for calcium and phosphorus in duplicate with the same methods employed for the analysis of the solution after equilibration; the values found were 7.8 mg. per cent for calcium and 3.1 mg. per cent for phosphorus.    As in the first set of

experiments, 100 mg. of $CaHPO_4$ were added to each test-tube. The results are given in Table II.

It is seen from Table II that here also equilibrium was attained after shaking for only 1 hour.    It is also seen that *the concentrations of both calcium and phosphorus were greater after equilibration than they were initially.*

If the conditions in the solution after shaking for such short

TABLE I.

*Equilibration of $H_2O$ with 100 Mg. of Crystalline $CaHPO_4$ at Room Temperature.*

| Solution. | Shaking time. | Ca | | P | | pH | $[Ca^{++}] \times [HPO_4^{=}]$ | $pK'_{s.p.}CaHPO_4$ |
|---|---|---|---|---|---|---|---|---|
| | hrs. | mg. per 100 cc. | mM | mg. per 100 cc. | mM | | $\times 10^7$ | |
| a | 1 | 3 4 | 0.85 | 3.2 | 1.03 | 6.9 | 5 5 | 6.26 |
| b | 2 | 3 6 | 0.90 | 3.2 | 1.03 | 6.9 | 5.9 | 6.23 |
| c | 3 | 3 8 | 0.95 | 3 2 | 1.03 | 6 9 | 6.2 | 6.21 |
| d | 4 | 3.6 | 0 90 | 3 3 | 1.06 | 6.8 | 5.4 | 6 27 |

TABLE II.

*Equilibration of Serum Solution with 100 Mg. of $CaHPO_4$ at Room Temperature.*

Initial Ca  = 7.8 mg. per 100 cc.
"    P  = 3.1  "    " 100  "
"    pH = 7.4

| Solution. | Shaking time. | Ca | | P | | pH | $[Ca^{++}] \times [HPO_4^{=}]$ | $pK'_{s.p.}CaHPO_4$ |
|---|---|---|---|---|---|---|---|---|
| | hrs. | mg. per 100 cc. | mM | mg. per 100 cc. | mM | | $\times 10^6$ | |
| a | 1 | 9.1 | 2.28 | 5.9 | 1.58 | 7.6 | 3.2 | 5.49 |
| b | 2 | 8.7 | 2.18 | 4.7 | 1.52 | 7.6 | 2 9 | 5.53 |
| c | 3 | 8 6 | 2.15 | 4.2 | 1.35 | 7.6 | 2.6 | 5 59 |
| d | 4 | 9.0 | 2.25 | 4.8 | 1.55 | 7.5 | 3.0 | 5.52 |
| e | 4 | 8.8 | 2 20 | 5.2 | 1.68 | 7.7 | 3.4 | 5.47 |

periods are genuine equilibrium conditions and not merely apparent ones, then they should be independent of the amount of solid phase present.    This was tested by repeating this set of experiments in an identical manner except that 1000 mg. of $CaHPO_4$ were added to each tube instead of 100 mg.    The results are given in Table III.

It is seen from Table III that the final concentrations of phosphorus were more than twice as great as the initial value.   The concentration of calcium decreased somewhat below the initial value with increasing shaking time; the phosphorus, however, increased as the calcium decreased.   Consequently the ion product $[Ca^{++}] \times [HPO_4^=]$ remained constant within the experimental error.   Here too, equilibrium was attained after shaking for only 1 hour, and the same equilibrium was reached as in the case where only one-tenth this amount of solid phase had been employed.

In the foregoing preliminary experiments, the pH determinations are given to only the first decimal place because their

<div align="center">

TABLE III.

*Equilibration of Serum Solution with 1000 Mg. of CaHPO₄ at Room Temperature*

Initial Ca  = 7 8 mg  per 100 cc.
"       P    = 3 1  "    "    100 "
"       pH = 7.4

</div>

| Solu-tion. | Shak-ing time. | Ca | | P | | pH | $[Ca^{++}] \times [HPO_4^=]$ | pK'$_s$ $_p$ CaHPO₄ |
|---|---|---|---|---|---|---|---|---|
| | hrs | mg per 100 cc | m M | mg per 100 cc | m M | | × 10⁶ | |
| a | 1 | 7 9 | 1 98 | 7 5 | 2 4 | 7 2 | 3 7 | 5 44 |
| b | 2 | 7 8 | 1 95 | 7 8 | 2 5 | 7 2 | 3 8 | 5 42 |
| c | 3 | | | 8 0 | 2 6 | 7 0 | | |
| d | 4 | 7 0 | 1 75 | 8 2 | 2 6 | 7 0 | 3 1 | 5 51 |

accuracy was not as great as that of the determinations in the subsequent experiments.   These preliminary experiments served to show: (1) a real equilibrium was attained after shaking for only 1 hour; (2) the ion product at equilibrium was independent of the amount of solid phase present; (3) the solutions appeared to act as if they were *undersaturated* with respect to secondary calcium phosphate.

### Equilibration Experiments at Room Temperature.

These preliminary experiments strikingly confirmed the conclusion which we (4) had previously drawn from our calculations of the ion product $[Ca^{++}] \times [HPO_4^=]$; namely, that "Inorganic serum

solutions with empirical Ca $\times$ P products less than 30 appear to be *undersaturated* with respect to $CaHPO_4$; calcification is not obtained with these solutions."

This is especially obvious in the results given in Table II. The initial Ca was 7.8 mg. per 100 cc.; after equilibration it *increased* in each instance to 8.6 mg. per cent or more. The initial phosphorus was 3.1 mg. per cent; after equilibration it *increased* in each case to 4.2 mg. per cent or more. These increases cannot be attributed to changes in pH. The changes in pH were small, the maximum change being from 7.4 to 7.7; the solutions all became slightly more alkaline. The effect of the change in pH would be to *reduce* the solubility of calcium phosphate. The increased concentrations of both calcium and phosphorus must therefore have been due to the fact that the solution was initially undersaturated with respect to $CaHPO_4$.

A series of experiments was then carried out to investigate further the findings of these preliminary experiments. A series of seven different inorganic serum solutions was prepared; all the solutions were made up to contain the same amount of calcium (8 mg. per cent) while the phosphorus was varied from 1 to 7 mg. per cent. The empirical Ca $\times$ P products in these seven solutions therefore varied from 8 to 56.

To about 20 cc. of each solution, 100 mg. of $CaHPO_4$ were added. The tubes were then sealed and the solutions were equilibrated for 1 hour at room temperature. The results are given in Table IV.

On comparing the initial and final Ca values in Columns 2 and 3 in Table IV it is seen that equilibration produced an increase in the calcium concentration of 2.2, 1.8, 1.0, and 0.2 mg. per cent respectively, in Solutions a, b, c, and d. Beginning with Solution e, it produced a decrease of 0.3, 0.9, and 1.3 mg. per cent respectively in the last three solutions. On comparing the initial and final phosphorus values in Columns 6 and 7, it is seen that as a result of the equilibration the phosphorus values increased 3.1, 2.5, 1.8, 1.2, and 0.5 mg. per cent respectively in the first five solutions; in Solutions f and g there was a decrease in the phosphorus concentration of 0.5 mg. per cent each. .

In all cases the changes in pH were slight and were toward the alkaline side. The increased concentrations of calcium and

TABLE IV.

*Equilibration of Serum Solutions with 100 Mg. of CaHPO₄ for 1 Hour at Room Temperature.*

| Solution. (1) | Ca | | [Ca⁺⁺] | | P | | pH | | [HPO₄⁼] | | Ca × P Initial. (12) | [Ca⁺⁺] × [HPO₄⁼] | | pK′$_{s.p.}$CaHPO₄ (15) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial. (2) | Final. (3) | Initial. (4) | Final. (5) | Initial. (6) | Final. (7) | Initial. (8) | Final. (9) | Initial. (10) | Final. (11) | | Initial. (13) | Final. (14) | |
| | *mg. per 100 cc.* | *mg. per 100 cc.* | *mM* | *mM* | *mg. per 100 cc.* | *mg. per 100 cc.* | | | *mM* | *mM* | | × 10⁶ | × 10⁶ | |
| a | 7.9 | 10.0 | | | 1.0 | 4 1 | | | | | | | | |
|   | 7.9 | 10.1 | 1.98 | 2 53 | 1 0 | 4 0 | 7.40 | 7.75 | 0 27 | 1.23 | 8 | 0.53 | 3.1 | 5.51 |
|   | 7.9 | 10.1 | | | 1.0 | 4.1 | | | | | | | | |
| b | 7.8 | 9.5 | | | 1.9 | 4.5 | | | | | | | | |
|   | 7.7 | 9.6 | 1.95 | 2 40 | 2 0 | 4 5 | 7.40 | 7.70 | 0.55 | 1.33 | 16 | 1.06 | 3.2 | 5.49 |
|   | 7.8 | 9.6 | | | 2.0 | 4.5 | | | | | | | | |
| c | 8.1 | 8 9 | | | 3 1 | 4 9 | | | | | | | | |
|   | 7.8 | 9 1 | 2 00 | 2 25 | 3 0 | 4 9 | 7.7 | 7.70 | 0.92 | 1.45 | 25 | 1.8 | 3.3 | 5.49 |
|   | 8 0 | 9.0 | | | 3.1 | 4.9 | | | | | | | | |
| d | 8.0 | 8 2 | | | 4 3 | 5 6 | | | | | | | | |
|   | 8.2 | 8.4 | 2.03 | 2.08 | 4 2 | 5 4 | 7 35 | 7.60 | 1.15 | 1.58 | 35 | 2.3 | 3.3 | 5.48 |
|   | 8.1 | 8.3 | | | 4.3 | 5.5 | | | | | | | | |
| e | 8.2 | 7.9 | | | 5.1 | 5.6 | | | | | | | | |
|   | 8.2 | 7.8 | 2.05 | 1.98 | 5 0 | 5 5 | 7.40 | 7.58 | 1.39 | 1.61 | 42 | 2.8 | 3.2 | 5.50 |
|   | 8.2 | 7.9 | | | 5.1 | 5.6 | | | | | | | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| f | 8 1 / 8 0 / 8.1 | 7 3 / 7 1 / 7.2 | 2 03 | 1 80 | 6 1 / 6 0 / 6 1 | 5 5 / 5 6 / 5 6 / 5.6 | 7 40 | 7 55 | 1 66 | 1 61 | 49 | 3 4 | 2 9 | 5 54 |
| g | 7 9 / 7 8 / 7 9 | 6 6 / 6 6 / 6 6 | 1 98 | 1 65 | 7 2 / 7 2 / 7.2 | 7 0 / 6 6 / 6 5 / 6 5 / 6.7 | 7 40 | 7 58 | 1 94 | 1 92 | 57 | 3 9 / Mean = 3.2 | 3.2 / 3.2 | 5 50 / 5 50 |

phosphorus which occurred in these solutions following equilibration cannot therefore be attributed to the changes in pH, since the effect of such pH changes would be to reduce the concentrations of calcium and phosphorus.

As a result of the equilibration, the ion product $[Ca^{++}] \times [HPO_4^-]$ increased sixfold in the case of Solution a, and threefold for Solution b.   There was an increase in the value of the ion product $[Ca^{++}] \times [HPO_4^-]$ in each of the first five solutions.   In Solution f with a Ca $\times$ P product of 49, the ion product showed a decrease after equilibration; in Solution g, with a Ca $\times$ P product of 57, the ion product showed a still larger decrease.

It is to be noted that in all seven solutions *the same value was obtained for $[Ca^{++}] \times [HPO_4^-]$ after equilibration.*   This shows that equilibration of serum solutions with crystalline $CaHPO_4$ for 1 hour results in the establishment of an equilibrium between the solid phase and the calcium, phosphate, and hydrogen ions in the solution.   Such serum solutions with empirical Ca $\times$ P products in the neighborhood of 50 are just saturated with respect to $CaHPO_4$; the smaller the initial value of the Ca $\times$ P product, the greater is the degree of *undersaturation* in the initial solutions. It is only solutions whose Ca $\times$ P products are greater than about 50 which are supersaturated; this is shown by the reduction in the value of the ion product $[Ca^{++}] \times [HPO_4^-]$ following equilibration of such solutions with the solid phase.

### *Equilibration Experiments at 38°.*

The foregoing experiments had been performed at room temperature, which was approximately 20°.   A series of experiments was next performed at 38° $\pm$ 0.5 to see whether similar results would be obtained at body temperature.   In the preceding set of experiments the initial calcium concentration was the same in all the solutions; *i.e.*, 8 mg. per cent.   The initial phosphorus values varied from 1 to 7 mg. per cent.   If a genuine equilibrium was being attained, then the same value for the ion product $[Ca^{++}] \times [HPO_4^-]$ should be obtained with greater initial calcium concentrations.   A set of inorganic serum solutions was accordingly made up in which the calcium concentration was 10 mg. per cent and in which the phosphorus concentration was varied from 2 to

TABLE V.

Equilibration of Serum Solutions with 100 Mg. of CaHPO₄ at 38°.

| Solution | Ca* | | [Ca⁺⁺] | | P* | | pH | | [HPO₄⁼] | | Ca × P Initial | [Ca⁺⁺] × [HPO₄⁼] | | pK's, p CaHPO₄ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial. | Final | Initial | Final | Initial | Final | Initial | Final | Initial | Final | | Initial. | Final | |
| | mg. per 100 cc | mg per 100 cc. | mM | mM | mg per 100 cc | mg per 100 cc | | | mM | mM | | ×10⁸ | ×10⁸ | |
| a | 10 3 | 9 3 | 2 58 | 2 33 | 2 0 | 4 3 | 7 55 | 7 60 | 0 57 | 1 24 | 21 | 1 5 | 2 9 | 5 54 |
| a′ | 5 1 | 7 2 | 1 28 | 1 80 | 4 0 | 6 7 | 7 41 | 7 60 | 1 09 | 1 93 | 20 | 1 4 | 3 5 | 5 46 |
| b | 10 3 | 7 6 | 2 58 | 1 90 | 3 1 | 5 1 | 7 35 | 7 58 | 0 83 | 1 47 | 32 | 2 1 | 2 8 | 5 55 |
| b′ | 5 0 | 6 0 | 1 25 | 1 50 | 6 2 | 8 1 | 7 39 | 7 56 | 1 68 | 2 32 | 31 | 2 1 | 3 5 | 5 46 |
| c | 9 9 | 9 9 | 2 48 | 2 48 | 4 1 | 5 5 | 7 25 | 7 45 | 1 05 | 1 52 | 41 | 2 6 | 3 8 | 5 42 |
| c′ | 5 2 | 5 5 | 1 30 | 1 38 | 8 0 | 9 4 | 7 36 | 7 55 | 2 14 | 2 70 | 42 | 2 8 | 3 7 | 5 43 |
| d | 10 1 | 8 2 | 2 53 | 2 05 | 5 2 | 5 5 | 7 57 | 7 58 | 1 48 | 1 58 | 53 | 3 8 | 3 2 | 5 49 |
| d′ | 5 1 | 4 7 | 1 28 | 1 18 | 10 0 | 10 8 | 7 35 | 7 53 | 2 68 | 3 10 | 51 | 3 4 | 3 7 | 5 43 |
| e | 10 2 | 8 2 | 2 55 | 2 05 | 6 4 | 6 0 | 7 25 | 7 57 | 1 68 | 1 73 | 65 | 4 3 | 3 5 | 5.46 / 5 47 |
| | | | | | | | | | | | | Mean = | 3 4 | |

* These figures are the averages of at least two determinations (cf. Table IV).

about 6 mg. per cent; the Ca × P products in these solutions therefore ranged from 20 to about 60.

At the same time another set of inorganic serum solutions was made up which was identical with the other set except that the initial calcium concentration in each solution was 5 mg. per cent. The initial phosphorus concentrations in the solutions of the second set varied from 4 to 10 mg. per cent; the Ca × P products therefore ranged from 20 to 50.

When the data are tabulated according to the value of the Ca × P product, as in Table V, it is seen that we had four pairs of solutions.   The Ca × P product of the solutions in each pair was the same; the calcium concentrations were in the ratio of 2 to 1, while the respective phosphorus concentrations were in the ratio of 1 to 2.

To about 20 cc. of each solution 100 mg. of crystalline CaHPO₄ were added.   The test-tubes were sealed and equilibrated continuously for 1 hour at room temperature in the shaking machine.   At the end of an hour, the temperature was raised to 38° in a water bath, and the solutions were placed in an air incubator at 38° for 1 hour; the solutions were shaken vigorously at 10 minute intervals during this 2nd hour.

The first pair of solutions given in Table V had Ca × P products of 21 and 20 and ion products, $[Ca^{++}] \times [HPO_4^-]$, of $1.5 \times 10^{-6}$ and $1.4 \times 10^{-6}$ respectively before equilibration; after equilibration the ion products were approximately twice as great.   The calcium of Solution a had decreased somewhat, while the phosphorus concentration had more than doubled; in Solution a′ the concentrations of both calcium and phosphorus increased markedly.

Solutions b and b′ had Ca × P products of 32 and 31 respectively, and ion products, $[Ca^{++}] \times [HPO_4^-]$, of $2.1 \times 10^{-6}$.   As a result of equilibration the values of this ion product increased in both cases by about 50 per cent.

Solutions c and c′ had Ca × P products of 41 and 42 and ion products, $[Ca^{++}] \times [HPO_4^-]$, of $2.6 \times 10^{-6}$ and $2.8 \times 10^{-6}$ respectively before equilibration.   After equilibration, the ion products were both greater; they were $3.8 \times 10^{-6}$ and $3.7 \times 10^{-6}$ respectively.

Solutions d and d′ had Ca × P products of 53 and 51, and ion products of $3.8 \times 10^{-6}$ and $3.4 \times 10^{-6}$ respectively.   After

equilibration the value of the ion product $[Ca^{++}] \times [HPO_4^-]$ was the same, within the experimental error, as the initial value for Solution d'. *This solution was therefore initially saturated with respect to CaHPO$_4$.*

In Solution d the ion product decreased from $3.8 \times 10^{-6}$ to $3.2 \times 10^{-6}$. Solution e with a Ca $\times$ P product of 65, had for the ion product $[Ca^{++}] \times [HPO_4^-]$ a value of $4.3 \times 10^{-6}$ before equilibration and a value of $3.5 \times 10^{-6}$ after equilibration. These were the only instances in which there was a definite decrease in this ion product; *these were the only solutions in this series which were initially supersaturated with respect to CaHPO$_4$.*

The mean of all the final values of the ion product in Table V is $3.4 \times 10^{-6}$. Inorganic serum solutions with Ca $\times$ P products of less than about 50 when shaken at 38° with crystalline CaHPO$_4$ show an increase in the value of the ion product $[Ca^{++}] \times [HPO_4^-]$; the lower the initial Ca $\times$ P product, the greater is the degree of undersaturation and consequently the greater is the difference between the initial and final values of the ion product $[Ca^{++}] \times [HPO_4^-]$. Such solutions with Ca $\times$ P products of about 50 are saturated with respect to CaHPO$_4$; equilibration produces no change in the value of the ion product. The only solutions in this series which showed decreases in the value of the ion product were those with Ca $\times$ P products of 53 and 65; these solutions were the only ones which were initially supersaturated. Hence for such inorganic serum solutions the dividing value between undersaturation and supersaturation is a Ca $\times$ P product of about 50.

As we have pointed out in a previous paper (4), there is a linear relation between this empirical product and the ion product of CaHPO$_4$. This relation is, at the pH of serum,

$$Ca \times P = 1.5 \times 10^7 \ ([Ca^{++}] \times [HPO_4^-])$$

Substitution of the solubility product of CaHPO$_4$ for $[Ca^{++}] \times [HPO_4^-]$ gives

$$Ca \times P = 1.5 \times 10^7 \times 3.4 \times 10^{-6} = 51$$

This border line value of 51, of the empirical Ca $\times$ P product, corresponds to the solubility product of CaHPO$_4$. In that earlier paper we recalculated the solubility product of CaHPO$_4$ from data in the literature. Extrapolation of the values of $K'_{s.p.}$CaHPO$_4$ to

the ionic strength of serum gave a value of $3.2 \times 10^{-6}$ with an estimated accuracy of about $\pm 0.8 \times 10^{-6}$; *i.e.*, from data in the literature, we calculated $K'_{s.p.}CaHPO_4$ to have a value at $38°$ of $(3.2 \pm 0.8) \times 10^{-6}$ at the ionic strength of serum.   It is seen from Table V that the actual determinations of $K'_{s.p.}CaHPO_4$ at the ionic strength of serum gave a value of $3.4 \times 10^{-6}$ at $38°$.

If the value of the ion product obtained after shaking for only 2 hours really is the equilibrium value for $CaHPO_4$, then it should be entirely independent of the initial concentrations of calcium and phosphorus.   Serum Solution a in Table VI was made so that the initial concentrations of calcium and phosphorus were both zero; after equilibration the ion product was the same as obtained with solutions which initially contained varying amounts of calcium and phosphorus.   Solution b contained initially only 1 mg. per cent each of calcium and phosphorus; after shaking with $CaHPO_4$ the same equilibrium value was obtained as for the previous solutions.   Thus the same equilibrium value is obtained for $[Ca^{++}] \times [HPO_4^-]$ whether the initial calcium concentration lies anywhere between 0 and 10 mg. per cent or whether the initial phosphorus concentration lies anywhere between 0 and 10 mg. per cent.

It will be noted that all of the solutions used so far were inorganic serum solutions which contained about 30 millimols of $CO_2$.   To see whether any marked difference would be obtained in solutions not containing any bicarbonate, Solution d was prepared without bicarbonate; it had the same calcium and phosphorus content as Solution c.   As a result of the equilibration, Solution c became more alkaline, as was expected; Solution d became more acid. The ion product $3.1 \times 10^{-6}$ obtained for Solution c was close to the mean value of $3.4 \times 10^{-6}$; the value $2.7 \times 10^{-6}$ obtained for Solution d was the lowest value so far obtained at $38°$.   This low value was apparently due to experimental error, for two other $CO_2$-free solutions after equilibration gave values of $3.3 \times 10^{-6}$ and $3.1 \times 10^{-6}$ (see Table VII).

Solutions e and f, both with initial calcium values of 5.1 mg. per cent, and initial phosphorus value of 8 and 10 mg. per cent respectively, were duplicates of Solutions c' and d' of Table V. These duplicates were slightly more acid than those of Table V; the equilibrium values for $[Ca^{++}] \times [HPO_4^-] \times 10^6$ were the

TABLE VI.

Equilibration of Serum Solutions with 100 Mg. of CaHPO₄ at 38°.

| Solution. | Ca* Initial. | Ca* Final. | [Ca⁺⁺] Initial. | [Ca⁺⁺] Final. | P* Initial. | P* Final. | pH Initial. | pH Final. | [HPO₄⁼] Initial. | [HPO₄⁼] Final. | Ca × P Initial. | [Ca⁺⁺] × [HPO₄⁼] Initial. $\times 10^4$ | [Ca⁺⁺] × [HPO₄⁼] Final. $\times 10^6$ | pK′$_{s.p.}$ CaHPO₄ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg. per 100 cc. | mg. per 100 cc. | mм | mм | mg. per 100 cc. | mg. per 100 cc. | | | mм | mм | | | | |
| a | 0.0 | 7.6 | 0.00 | 1 90 | 0 0 | 6.9 | 7.38 | 7 59 | 0.00 | 1 98 | 0 | 0.0 | 3.8 | 5.42 |
| b | 1.2 | 8.2 | 0 30 | 2.05 | 1 0 | 7.1 | 7 05 | 7 25 | 0.23 | 1 81 | 1 | 0 07 | 3.7 | 5.42 |
| c | 10.1 | 9.1 | 2 53 | 2.28 | 3 0 | 4 8 | 7.24 | 7.54 | 0.76 | 1 36 | 30 | 1.9 | 3.1 | 5.51 |
| d† | 10.2 | 11.7 | 2 55 | 2.93 | 3 2 | 5 0 | 7.12† | 6 80 | 0.76 | 0 93 | 33 | 1 9 | 2.7 | 5.57 |
| e | 5.1 | 5.4 | 1.28 | 1.35 | 8.0 | 9.5 | 7.22 | 7 42 | 2.01 | 2 59 | 41 | 2 6 | 3.5 | 5.46 |
| f | 5.1 | 4.8 | 1.28 | 1.20 | 10.0 | 10.7 | 7.10 | 7 33 | 2 36 | 2.83 | 51 | 3.0 | 3.4 | 5.47 |
| | | | | | | | | | | | | Mean = | 3.4 | 5.48 |

* These figures are the averages of at least two determinations (cf. Table IV).
† Contained no NaHCO₃ or CO₂.

TABLE VII.

Equilibration of Serum Solutions with 1000 Mg. of CaHPO₄ at 38°.

| Solution. | Ca* Initial. | Ca* Final. | [Ca⁺⁺] Initial. | [Ca⁺⁺] Final. | P* Initial. | P* Final. | pH Initial. | pH Final. | [HPO₄⁼] Initial. | [HPO₄⁼] Final. | Ca × P Initial. | [Ca⁺⁺] × [HPO₄⁼] Initial. $\times 10^4$ | [Ca⁺⁺] × [HPO₄⁼] Final. $\times 10^4$ | pK′$_{s.p.}$ CaHPO₄ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg. per 100 cc. | mg. per 100 cc. | mм | mм | mg. per 100 cc. | mg. per 100 cc. | | | mм | mм | | | | |
| a | 10.1 | 6.9 | 2.53 | 1.73 | 3 0 | 9.1 | 7.25 | 7.15 | 0.77 | 2.2 | 30 | 1.9 | 3.8 | 5.42 |
| a′ | 10.1 | 7.1 | 2.53 | 1.78 | 3 0 | 9.0 | 7.25 | 7.10 | 0.77 | 2.1 | 30 | 1.9 | 3.8 | 5.42 |
| b† | 10.2 | 15.7 | 2 55 | 3.93 | 3.2 | 12.4 | 7.27 | 6.10 | 0.83 | 0.85 | 33 | 2.1 | 3.3 | 5.48 |
| b′† | 10.2 | 15.0 | 2.55 | 3.75 | 3.2 | 12.3 | 7.27 | 6.10 | 0.83 | 0.83 | 33 | 2.1 | 3.1 | 5.51 |

* These figures are the averages of at least two determinations (cf. Table IV),
† Solutions b and b′ contained no bicarbonate or CO₂.

same however; values of 3.7 and 3.7 were obtained for Solutions c' and d' of Table V and values of 3.5 and 3.4 were obtained for Solutions e and f of Table VI.   Thus the equilibrium obtained is readily and accurately reproducible.

The last set of equilibration experiments is given in Table VII. The solutions in this set were all equilibrated with 1000 mg. of CaHPO₄ instead of with the customary 100 mg. which we had employed in all the other experiments at 38°; in all the experiments at 38° the equilibration was performed as described on p. 708.   As in experiments performed at room temperature (see Table III), the same equilibrium value was obtained at 38° whether 100 mg. or 1000 mg. of crystalline CaHPO₄ were employed.   The equilibrium value obtained is therefore independent of the amount of solid phase present.

Solutions a and a' were aliquots of the same serum solution. Inspection of Table VII shows that the equilibrium values for calcium, phosphorus, and pH, and therefore for $pK'_{s.p.}$.CaHPO₄ were almost identical.   The same conclusion is reached from inspection of the equilibrium values for Solutions b and b', which were aliquots of a second solution.   This second solution was CO₂-free; it contained neither bicarbonate nor CO₂.   The analyses made after equilibration showed a 50 per cent increase in calcium, and a 400 per cent increase in phosphorus in Solutions b and b'; it seemed as if the ion product of CaHPO₄ was not applicable to such solutions.   However, calculation showed that the same values for $[Ca^{++}] \times [HPO_4^=]$ were obtained in those two solutions as had been obtained in all the other solutions.   Solutions b and b' had become decidedly more acid as a result of the equilibration; the concentration of total phosphorus therefore increased enormously in order that a concentration of $[HPO_4^=]$ be obtained sufficient for the ion product $[Ca^{++}] \times [HPO_4^=]$ to be equal to $K'_{s.p.}$.CaHPO₄.   This brought out strikingly the importance of $K'_{s.p.}$.CaHPO₄ as the determining quantity in the equilibrium.

The results obtained at room temperature are summarized in Table VIII.   The values of the ion product $[Ca^{++}] \times [HPO_4^=]$ obtained after equilibration with crystalline CaHPO₄ gave a mean value of $3.2 \times 10^{-6}$ with an average deviation of $\pm 0.2 \times 10^{-6}$; the A.D. was therefore less than $\pm 0.1 \times 10^{-6}$.   Since the same value for the ion product $[Ca^{++}] \times [HPO_4^=]$ was obtained

TABLE VIII.

Summary of Equilibration Experiments at Room Temperature.

| Experiment No. | Initial $Ca° \times P°$ | Initial $[Ca^{++}] \times [HPO_4 =]$ | $K'_{s.p.}$ CaHPO$_4$ | Deviation | $pK'_{s.p.}$ CaHPO$_4$ | Deviation |
|---|---|---|---|---|---|---|
|  |  | $\times 10^6$ | $\times 10^6$ | $\times 10^6$ |  |  |
| II a | $8 \times 3 = 24$ | 1.7 | 3.2 | 0.0 | 5.49 | $-0.01$ |
| II b | $8 \times 3 = 24$ | 1.7 | 2.9 | $-0.3$ | 5.53 | $+0.03$ |
| II c | $8 \times 3 = 24$ | 1.7 | 2.6 | $-0.6$ | 5.59 | $+0.06$ |
| II d | $8 \times 3 = 24$ | 1.7 | 3.0 | $-0.2$ | 5.52 | $+0.02$ |
| II e | $8 \times 3 = 24$ | 1.7 | 3.4 | $+0.2$ | 5.47 | $-0.03$ |
| III a | $8 \times 3 = 24$ | 1.7 | 3.7 | $+0.5$ | 5.44 | $-0.06$ |
| III b | $8 \times 3 = 24$ | 1.7 | 3.8 | $+0.6$ | 5.42 | $-0.08$ |
| III d | $8 \times 3 = 24$ | 1.7 | 3.1 | $-0.1$ | 5.51 | $+0.01$ |
| IV a | $8 \times 1 = 8$ | 0.5 | 3.1 | $-0.1$ | 5.51 | $+0.01$ |
| IV b | $8 \times 2 = 16$ | 1.1 | 3.2 | 0.0 | 5.49 | $-0.01$ |
| IV c | $8 \times 3 = 24$ | 1.8 | 3.3 | $+0.1$ | 5.49 | $-0.01$ |
| IV d | $9 \times 4 = 32$ | 2.3 | 3.3 | $+0.1$ | 5.48 | $-0.02$ |
| IV e | $8 \times 5 = 40$ | 2.8 | 3.2 | 0.0 | 5.50 | 0.00 |
| IV f | $8 \times 6 = 48$ | 3.4 | 2.9 | $-0.3$ | 5.54 | $+0.04$ |
| IV g | $8 \times 7 = 56$ | 3.9 | 3.2 | 0.0 | 5.50 | 0.00 |
|  |  |  | Mean = 3.2 | a.d. $= \pm 0.2$<br>A.D. $= \pm 0.1$ | Mean = 5.50 | a.d. $= \pm 0.03$<br>A.D. $= \pm 0.01$ |

At room temperature $K'_{s.p.}$ CaHPO$_4$ = $(3.2 \pm 0.1) \times 10^{-6}$.
$pK'_{s.p.}$ CaHPO$_4$ = $5.50 \pm 0.01$.

\* Concentrations are expressed in mg. per 100 cc. to the nearest whole number.

TABLE IX.

Summary of Equilibration Experiments at 38°.

| Experiment No. | Initial. Ca* × P* | Initial [Ca⁺⁺] × [HPO₄=] | $K'_{s.p.}$ CaHPO₄ | Deviation | $pK'_{s.p.}$ CaHPO₄ | Deviation |
|---|---|---|---|---|---|---|
| | | $\times 10^5$ | $\times 10^5$ | | | |
| V a | 10 × 2 = 20 | 1.5 | 2.9 | −0.5 | 5.54 | +0.07 |
| V a' | 5 × 4 = 20 | 1.4 | 3.5 | +0.1 | 5.46 | −0.01 |
| V b | 10 × 3 = 30 | 2.1 | 2.8 | −0.6 | 5.55 | +0.08 |
| V b' | 5 × 6 = 30 | 2.1 | 3.5 | +0.1 | 5.46 | −0.01 |
| V c | 10 × 4 = 40 | 2.6 | 3.8 | +0.4 | 5.42 | −0.05 |
| V c' | 5 × 8 = 40 | 2.8 | 3.7 | +0.3 | 5.43 | −0.04 |
| V d | 10 × 5 = 50 | 3.8 | 3.2 | −0.2 | 5.49 | +0.02 |
| V d' | 5 × 10 = 50 | 3.4 | 3.7 | +0.3 | 5.43 | −0.04 |
| V e | 10 × 6 = 60 | 4.3 | 3.5 | +0.1 | 5.46 | −0.01 |
| VI a | 0 × 0 = 0 | 0.0 | 3.8 | +0.4 | 5.42 | −0.05 |
| VI b | 1 × 1 = 1 | 0.1 | 3.7 | +0.3 | 5.42 | −0.05 |
| VI c | 10 × 3 = 30 | 1.9 | 3.1 | −0.3 | 5.51 | +0.04 |
| VI d | 10 × 3 = 30† | 1.9 | 2.7 | −0.7 | 5.57 | +0.10 |
| VI e | 5 × 8 = 40 | 2.6 | 3.5 | +0.1 | 5.46 | −0.01 |
| VI f | 5 × 10 = 50 | 3.0 | 3.4 | 0.0 | 5.47 | 0.00 |
| VII a | 10 × 3 = 30 | 1.9 | 3.8 | +0.4 | 5.42 | −0.05 |
| VII a' | 10 × 3 = 30 | 1.9 | 3.8 | +0.4 | 5.42 | −0.05 |
| VII b | 10 × 3 = 30† | 2.1 | 3.3 | −0.1 | 5.48 | +0.01 |
| VII b' | 10 × 3 = 30† | 2.1 | 3.1 | −0.3 | 5.51 | +0.04 |
| | | | Mean = 3.4 | a.d. = ±0.3 <br> A.D. = ±0.1 | Mean = 5.47 | a.d. = ±0.04 <br> A.D. = ±0.01 |

At 38° $K'_{s.p.}$ CaHPO₄ = $(3.4 \pm 0.1) \times 10^{-6}$.

$pK'_{s.p.}$ CaHPO₄ = $5.47 \pm 0.01$.

* Concentrations are expressed in mg. per 100 cc. to the nearest whole number.

† These solutions contained no NaHCO₃ or CO₂.

in all solutions which had been shaken with crystalline $CaHPO_4$, regardless of the initial calcium or initial phosphorus concentrations, equilibrium had obviously been reached; the value of $(3.2 \pm 0.1) \times 10^{-6}$ is therefore $K'_{s.p.}CaHPO_4$, the solubility product of $CaHPO_4$ at $\sqrt{\mu} = 0.38$. When expressed as negative logarithms these results give a mean value of $5.50 \pm 0.01$ for $pK'_{s.p.}CaHPO_4$.

The results obtained at $38°$ are summarized in Table IX. The results were quite similar to those obtained at room temperature. $K'_{s.p.}CaHPO_4$ was found to be $(3.4 \pm 0.1) \times 10^{-6}$; $pK'_{s.p.}CaHPO_4$ was therefore $5.47 \pm 0.01$.

<center>DISCUSSION.</center>

These experiments show clearly that inorganic serum solutions can rapidly be brought into equilibrium with crystalline $CaHPO_4$. The ion product $[Ca^{++}] \times [HPO_4^=]$ obtained at equilibrium was a constant,—$(3.2 \pm 0.1) \times 10^{-6}$ at room temperature, and $(3.4 \pm 0.1) \times 10^{-6}$ at $38°$. The latter value is in good agreement with the value of $3.2 \times 10^{-6}$ which we had previously obtained by calculation from data in the literature. This value, which was obtained by extrapolation to the ionic strength of serum, had an estimated accuracy of 25 per cent; i.e., we assigned to $K'_{s.p}CaHPO_4$ a value of $(3.2 \pm 0.8) \times 10^{-6}$ at $38°$. The mean value which we have obtained experimentally at the ionic strength of serum is $3.4 \times 10^{-6}$ with an A.D. of $\pm 0.1 \times 10^{-6}$; the mean value is therefore correct to within about 3 per cent.

These experiments show clearly: (1) solutions having the inorganic composition of ricketic serum are decidedly undersaturated with respect to $CaHPO_4$; (2) solutions having the inorganic composition of normal serum are either very slightly undersaturated or are just saturated with respect to $CaHPO_4$; (3) it is only solutions which have empirical $Ca \times P$ products greater than about 50 that are supersaturated with respect to $CaHPO_4$.

It is generally believed that a fraction of the serum calcium is bound to protein in an unionized or slightly ionized compound. If this is true, then $[Ca^{++}]$ will be less than $[Ca]$. The ion product $[Ca^{++}] \times [HPO_4^=]$ in a serum with a given calcium and phosphorus

content cannot be greater than the ion product in an inorganic serum solution with the same calcium and phosphorus content. If it is at all different, the ion product must be less in serum since part of the calcium appears to be bound to protein.   Inorganic serum solutions with empirical $Ca \times P$ products of less than about 50 are undersaturated with respect to $CaHPO_4$; ricketic serum would therefore appear to be undersaturated with respect to this substance.

Mond and Netter (11) attempted to account for what they considered the stable supersaturation of serum with respect to calcium.   Nitschke (12) and Nitschke and Freyschmidt (13) have also studied "die Zustandsform des Calciums im Serum." In his latest article Nitschke (14) obtained

$$[Ca^{++}] \times [HPO_4^{=}] = K = 6 7 \times 10^{-6}$$

as the value of the equilibrium constant in systems which he regarded as supersaturated with respect to calcium phosphate. Marrack and Thacker (15) found that a 0.9 per cent sodium chloride solution at about pH 7.4 which contained 3.1 mg. per cent of phosphorus and 10 mg. per cent of calcium remained unchanged for weeks; no precipitate formed and no change in pH occurred.   They concluded that "the calcium remains as ion in such supersaturated solutions until precipitation occurs." Kleinman (16) considered that his data constituted "den Beweis für den Übersättigungszustand" of serum.

In view of the results of our equilibration experiments the question is raised as to whether the systems studied in such investigations as the foregoing are supersaturated.   If the ion products $[Ca^{++}] \times [HPO_4^{=}]$ in such systems are compared with the value of $K'_{s.p.}.CaHPO_4$ obtained for the ionic strength of serum, explanations of the experimental findings in such studies as the foregoing may be reached which are in better accord with the general theory of equilibria in saturated and supersaturated solutions.

We realize of course that the presence of $CaHPO_4$ in bones has never been demonstrated.   It is generally believed that the bone phosphate is $Ca_3(PO_4)_2$.   When, however, the solubility product of tricalcium phosphate is employed, the mechanism of calcification becomes more obscured rather than clarified.   Taylor and

Sheard (17) have recently suggested that "The solubility product of such a substance as podolite, $3Ca_3(PO_4)_2 \cdot CaCO_3$, therefore should be used rather than that of brushite $(CaHPO_4 \cdot 2H_2O)$ or of the compound $Ca_3(PO_4)_2$."

Our (18) analyses have shown that the composition of bone cannot be represented by the formula $3Ca_3(PO_4)_2 \cdot CaCO_3$. According to this formula

$$\frac{\text{Carbonate Ca}}{\text{Total Ca}} = \frac{1}{10}$$

*i.e.*, the carbonate calcium constitutes exactly 10 per cent of the total calcium.

For normal rat bone we found that the ratio carbonate Ca:total Ca varies all the way from 8 per cent in young rats to 16 per cent in mature rats.   For human pathological calcification we (19) obtained values of this ratio which varied from 13 per cent to 17 per cent.   Obviously the composition of bone is not constant; the ratio carbonate Ca:total Ca varies with age, and it is different for normal and ricketic animals of the same age.   Neither is this ratio constant in pathological calcification.   There appear to be at least two inorganic calcium compounds in bone; the amount of one of these compounds present in a given bone is independent of the amount of the other compound.   The relative amount of $CaCO_3$ present in some bones is *less* than that represented by the formula $3 Ca_3(PO_4)_2 \cdot CaCO_3$; in other bones the amount of $CaCO_3$ present is considerably greater than that given by this formula.

It is possible to conceive of bone, as Taylor and Sheard have done, as consisting of podolite or dahllite plus a variable amount of excess $CaCO_3$.   This would account for the composition of such bones as have a carbonate Ca:total Ca ratio greater than 10 per cent.   This theory however, fails to account for such bones as have a ratio of less than 10 per cent; in these bones there is insufficient $CaCO_3$ to fit the formula for podolite.   The assumption would then have to be made that such bones consist of podolite plus a variable amount of excess $Ca_3 (PO_4)_2$.

If for the moment we grant the generally accepted view that $Ca_3(PO_4)_2$ is a definite molecular species, then the only formula which represents the analyses is $x\, Ca_3(PO_4)_2 + y\, CaCO_3$.   This,

however, is not a compound but a mixture of two compounds in varying proportions.[2]

In a previous communication last year we (4) stated that the presence of $Ca_3(PO_4)_2$ in bone has not yet been demonstrated, and that the preparation of specimens of pure tricalcium phosphate has never been reported.   Jacob and Reynolds (20) and Lorah, Tartar, and Wood (21) have recently reported results which agree with those of previous investigators; they were unable to prepare precipitates of tertiary calcium phosphate which give the theoretical Ca:P ratio on analysis.   So far as we are aware, no new evidence has appeared since then which definitely establishes the presence of $Ca_3(PO_4)_2$ in bone.

The only evidence for the presence of tricalcium phosphate in bone is the reported constancy of the ratio residual Ca: residual P in the vicinity of 1.94; this is the ratio calculated from the formula $Ca_3(PO_4)_2$.   Certainly the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ and $K'_{s.p.}Ca_3(PO_4)_2$ do not account satisfactorily for either calcification or for precipitation.   It is not impossible that bone contains a mixture of $CaHPO_4$ and $Ca(OH)_2$, or a basic secondary calcium phosphate.   The close approximation of the Ca:P ratio to 1.94 might be equally well explained on the *assumption* that bone contains $2 CaHPO_4 + CaO$ as well as on the *assumption* that it contains $Ca_3(PO_4)_2$.

*We regard the composition of bone as an open question.*   We do not know whether $CaHPO_4$ is present in bone.   But the correspondence between these equilibration experiments on the one hand and calcification *in vitro* and rickets on the other hand are highly suggestive.   In future investigations dealing with the determination of the nature of the compounds present in bone generally, and in primary calcification especially, the possibility of the presence of $CaHPO_4$ should not be overlooked.

### SUMMARY.

1. When distilled water is shaken with crystalline $CaHPO_4$, equilibrium is attained at the end of 1 hour.

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[2] This discussion refers to analyses in which the ratio residual Ca: residual P is 1.94.   In the case of primary calcification even this formula would not apply (18).   For primary calcification the analogous formula would appear to be $x Ca_3 (PO_4)_2 + y CaCO_3 + z CaX$.

2. This equilibrium is maintained unchanged during a subsequent equilibration period of 3 hours.

3. When inorganic serum solutions are shaken with crystalline $CaHPO_4$, equilibrium is attained at the end of 1 hour.

4. The ion product $[Ca^{++}] \times [HPO_4^{=}]$ at equilibrium is independent of the amount of excess solid phase.

5. The value of the ion product, $[Ca^{++}] \times [HPO_4^{=}]$, in inorganic serum solutions after equilibration with $CaHPO_4$ is a constant.

6. This constant is independent of the initial concentrations of calcium and phosphorus.

7. Fifteen experiments with various inorganic serum solutions gave a mean value of $(3.2 \pm 0.1) \times 10^{-6}$ for $K'_{s.p.}CaHPO_4$ at room temperature.   This gives a mean value of $5.50 \pm 0.01$ for $pK'_{s.p.}CaHPO_4$.

8. Nineteen experiments with various inorganic serum solutions gave a mean value of $(3.4 \pm 0.1) \times 10^{-6}$ for $K'_{s.p.}CaHPO_4$ at $38°$; this gives a mean value of $5.47 \pm 0.01$ for $pK'_{s.p.}CaHPO_4$.

9. Inorganic serum solutions with empirical $Ca \times P$ products of less than about 50 are undersaturated with respect to $CaHPO_4$; those with $Ca \times P$ products of about 50 are just saturated; it is only solutions with $Ca \times P$ products greater than about 50 which are supersaturated with respect to $CaHPO_4$.

10. The bearing of these experiments on calcification is briefly discussed.

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# COMPOSITION OF BONE.

## VIII. CONDUCTIVITY TITRATIONS OF CALCIUM ION WITH CHLORIDE, ACETATE, LACTATE, AND CITRATE IONS AT 38°.*

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Shipley, Kramer, and Howland (1) in their experiments on calcification *in vitro* found that when the calcium was present as chloride, acetate, or lactate, calcification was prompt and thorough; when the calcium was present as citrate no calcification was obtained. In previous communications we (2, 3) discussed the rôle of calcium citrate in calcification and presented conductivity titration data which gave direct evidence for the binding of calcium ions by sodium citrate in some sort of soluble, slightly ionized or unionized complex. Those experiments were performed at 25°. The present paper gives the results of similar titrations with sodium citrate performed at 38°, together with the results obtained with sodium acetate and sodium lactate.

## Materials and Apparatus.

The instruments employed were the same as those described in our previous experiments. The temperature was kept at 38.0°; the variation was less than $\pm$ 0.1°. The cells were large Pyrex test-tubes about 20 cm. long and 4 cm. in diameter. Removable electrodes were used as before, except that the squares of platinum foil were fixed in a vertical position instead of in a horizontal position.

Two stock calcium solutions were prepared. In one case the calculated weight of $CaCO_3$ was decomposed with just enough

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* These experiments were reported at a meeting of the Chemistry Research Club, New York, April 4, 1929.

dilute HCl to cause it to dissolve.   After solution was complete, the $CO_2$ was removed by boiling; the solution was then cooled and made up to volume.   In the second case, calcium oxide was prepared by heating calcium oxalate in a platinum dish; it was then dissolved in water and made up to volume.   The various solutions of 0.005 N Ca were made by dilution of aliquots of these two solutions.   In some instances the aliquots were made up to volume with water only; in others, HCl or NaOH was added until the pH of the 0.005 N Ca solution was adjusted to 7.4.

All the salts used were of the analyzed reagent grade; the soluble salts were recrystallized at least once and dried to constant weight. The KCl used in determining the cell constant was recrystallized three times.   Fused NaCl was used; the aqueous solutions gave a neutral reaction.   In making the 0.005 N sodium acetate solution the formula $NaC_2H_3O_2 \cdot 3H_2O$ was employed; for sodium citrate the formula $Na_3C_6H_5O_7 \cdot 2H_2O$ was used.[1]   The sodium lactate was prepared from lactic acid.

The water used in making up the solutions was allowed to come to equilibrium with the air;[2] the specific conductance of the water when used varied in general from about 2.0 to 4.0 $\times$ $10^{-6}$ mhos.

### Experiment 1.   Titration of $CaCl_2$ with NaCl.

The specific conductance of the solution of 0.005 N $CaCl_2$ whose pH had been adjusted to 7.4 with NaOH was found to be 9.23 $\times$ $10^{-4}$ mhos, and that of the 0.005 N NaCl solution to be 8.41 $\times$ $10^{-4}$ mhos.   In the titration 20.0 cc. of the $CaCl_2$ were put into the cell and the NaCl solution was added 4.0 cc. at a time until the total volume was 64.0 cc.   The specific conductance was determined about 10 minutes after each addition.   The observed results are given in Table I and by the solid line in Fig. 1.

The calculated minimum conductances of the various mixtures of the $CaCl_2$ and NaCl solutions were obtained according to the simple rule of mixtures, using the expression

$$\text{Calculated specific conductance} = \frac{v_1c_1 + v_2c_2}{v_1 + v_2} \qquad (1)$$

[1] Murray, B. L., Standards and tests for reagent and C. P. chemicals, New York (1927).

[2] Cf. statement regarding "equilibrium water" by Partington, J. R., in Taylor, H. S., A treatise on physical chemistry, New York, 1, 522 (1924).

where $v_1$ and $v_2$ are the volumes of the solutions mixed and $c_1$ and $c_2$ are the specific conductances of the original solutions, *i.e.* $9.23 \times 10^{-4}$ and $8.41 \times 10^{-4}$ mhos respectively. These calculated minimum values are given graphically by the lower broken line, Curve B, in Fig. 1.

The effect of the increase in dilution on the specific conductance of each salt was determined empirically by means of dilution titrations as described in a previous paper (3). The conductances of the original NaCl and $CaCl_2$ solutions, "corrected" for each dilu-

TABLE I.

*Titration of $CaCl_2$ with NaCl.*

Initial solution, 20.0 cc. of 0.005 N $CaCl_2$.

| 0 005 N NaCl added. | Total volume | Specific conductance of mixture. |
|---|---|---|
| *cc* | *cc* | *mhos* $\times 10^4$ |
| 0 0 | 20 0 | 9 23 |
| 4 0 | 24 0 | 9 35 |
| 8 0 | 28 0 | 9 32 |
| 12 0 | 32 0 | 9 23 |
| 16 0 | 36 0 | 9 23 |
| 20 0 | 40 0 | 9 10 |
| 24 0 | 44 0 | 9 10 |
| 28 0 | 48 0 | 9 04 |
| 32 0 | 52 0 | 9 01 |
| 36 0 | 56 0 | 9 01 |
| 40 0 | 60 0 | 8 98 |
| 44 0 | 64 0 | 8 92 |

Specific conductance of the 0 005 N NaCl solution = $8 41 \times 10^{-4}$ mhos.

tion, were calculated from the dilution titrations in a manner similar to that employed in the earlier work; *i.e.*, it was obtained from the expression

$$\text{"corrected" specific conductance} = \frac{v_1 + v_2}{v_1} \times \text{observed specific conductance}$$

The calculated maximum conductances of the mixtures were also obtained from Equation 1; however, the specific conductances of the original solutions used for each mixture case were the values "corrected" for that dilution instead of the experimentally ob-

served values of the specific conductances of the original solutions. For each mixture a different pair of "corrected" values, obtained by means of Equation 2, was used for $c_1$ and $c_2$. This calculated maximum curve is the upper broken line, Curve A, in Fig. 1.

It is seen from Fig. 1 that the observed conductances are about midway between the calculated minimum values and the calculated maximum values. The dot and dash line shows the specific conductance of the 0.005 N NaCl solution; the observed and calculated curves are all above this line.
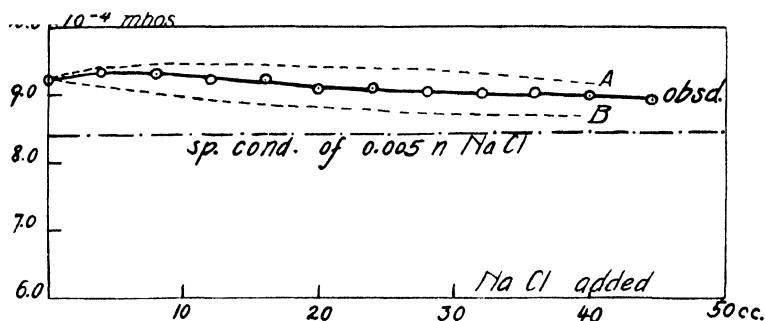


Fig. 1. Titration of 0.005 N CaCl₂ with 0.005 N NaCl. Curve A indicates· calculated maximum; Curve B, calculated minimum.

*Experiment 2. Titration of CaCl₂ with Sodium Acetate.*

In this experiment, as well as in Experiment 1, the 0.005 N CaCl₂ solution was made by diluting an aliquot of the stock CaCl₂ solution prepared from $CaCO_3$. The specific conductance of this solution after adjusting the pH to 7.4 was $8.92 \times 10^{-4}$ mhos and that of the 0.005 N sodium acetate solution was found to be $5.62 \times 10^{-4}$ mhos. The results of this conductivity titration are given in Table II and are given graphically by the solid line in Fig. 2. The calculated minimum curve is the lower broken line, Curve B, in Fig. 2.

The effect of dilution of 0.005 N sodium acetate was allowed for as described in the preceding experiment; the maximum conductances calculated with the aid of these data are given by the upper broken line, Curve A, in Fig. 2.

It is seen from Fig. 2 that the observed specific conductances

are intermediate between the calculated maximum and the calculated minimum values. The dot and dash line, Curve C,

TABLE II.

*Titration of CaCl₂ with Na Acetate.*

Initial solution, 20.0 cc. of 0.005 N CaCl₂.

| 0.005 Na acetate added. | Total volume. | Specific conductance of mixture. |
|:---:|:---:|:---:|
| *cc.* | *cc.* | *mhos $\times$ 10⁴* |
| 0 0 | 20 0 | 8.92 |
| 4 0 | 24 0 | 8 61 |
| 8 0 | 28 0 | 8.33 |
| 12.0 | 32 0 | 7.99 |
| 16 0 | 36 0 | 7.78 |
| 20 0 | 40.0 | 7.59 |
| 24 0 | 44 0 | 7.43 |
| 28 0 | 48 0 | 7.31 |
| 32.0 | 52 0 | 7 25 |
| 36 0 | 56.0 | 7.21 |
| 40 0 | 60 0 | 7.08 |
| 44.0 | 64.0 | 7.10 |

Specific conductance of the 0.005 N Na acetate solution    5.62 $\times$ 10⁻⁴ mhos.
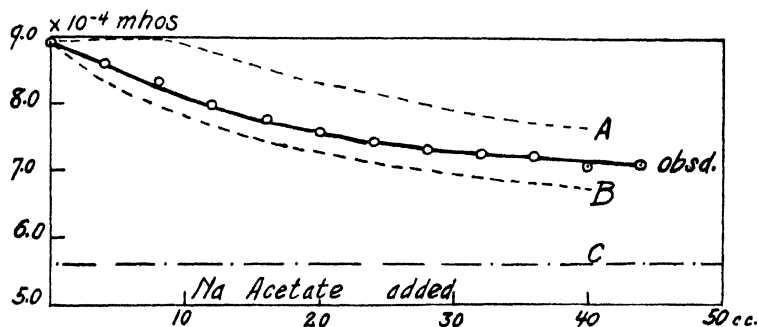


FIG. 2. Titration of 0.005 N CaCl₂ with 0.005 N Na acetate. Curve A indicates calculated maximum; Curve B, calculated minimum; Curve C, specific conductance of 0.005 N Na acetate.

shows the specific conductance of the 0.005 N sodium acetate solution; the observed and calculated curves are all above this line; and they all slope down toward it.

*Experiment 3.    Titration of CaCl₂ with Sodium Lactate.*

Another aliquot of the $CaCl_2$ solution prepared from $CaCO_3$ had a specific conductance of 8.51 $\times$ $10^{-4}$ mhos after adjusting the

TABLE III.

*Titration of CaCl₂ with Na Lactate.*

Initial solution, 20.0 cc. of 0.005 N $CaCl_2$.

| 0.005 N Na lactate added | Total volume | Specific conductance of mixture. |
|---|---|---|
| cc | cc | mhos $\times 10^4$ |
| 0 0 | 20 0 | 8 51 |
| 4 0 | 24.0 | 8 22 |
| 8 0 | 28 0 | 8 04 |
| 12 0 | 32 0 | 7.58 |
| 16 0 | 36 0 | 7 39 |
| 24 0 | 44 0 | 7.00 |
| 32 0 | 52.0 | 6.80 |
| 40 0 | 60.0 | 6.60 |
| 44 0 | 64 0 | 6.53 |
| 48 0 | 68.0 | 6 49 |

Specific conductance of the 0.005 N Na lactate solution = 5.35 $\times$ $10^{-4}$ mhos
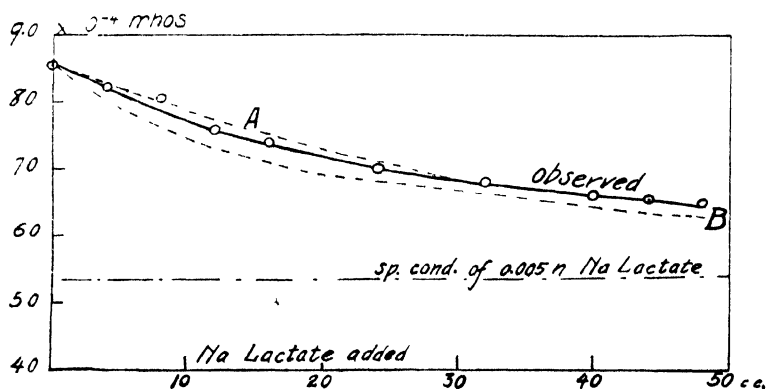


Fɪɢ. 3   Titration of 0.005 N $CaCl_2$ with 0.005 N Na lactate.   Curve A indicates calculated maximum; Curve B, calculated minimum.

pH to 7.4.   A 20.0 cc. aliquot of this 0.005 N $CaCl_2$ solution was titrated with a 0.005 N sodium lactate solution which had a specific

conductance of $5.35 \times 10^{-4}$ mhos.    The results are given in Table III and by the solid line in Fig. 3.

The calculated minimum curve is the broken lower one, Curve B, in this figure.    The maximum conductances calculated with the aid of dilution titrations are given by the upper broken line, Curve A, of Fig. 3.

It will be seen that the first part of the experimental curve lies between the calculated maximum and the calculated minimum curves; the experimental curve lies close to the calculated maximum curve and, after the addition of 30.0 cc. of sodium lactate, is identical with it.    The dot and dash line shows the specific conductance of the 0.005 N sodium lactate solution; the observed and calculated curves are all above this line, and they all slope down towards it.

*Experiment 4.    Titration of CaCl$_2$ with Sodium Citrate.*

Another 0.005 N CaCl$_2$ solution, made by diluting another aliquot of the same stock CaCl$_2$ solution prepared from CaCl$_3$ gave, after adjustment to pH 7.4, a specific conductance of $9.10 \times 10^{-4}$ mhos.    This was titrated with a 0.005 N solution of sodium citrate which had a specific conductance of $6.80 \times 10^{-4}$ mhos.    The results are given in Table IV.

In Fig. 4 the solid line gives the experimental results, while the lower broken line gives the calculated minimum values.    The upper broken line gives the calculated maximum values.

It is seen from Fig. 4 that the calculated maximum and minimum curves are similar to those in the preceding three experiments.    They both slope down towards the dot and dash line, which represents the specific conductance of the 0.005 N sodium citrate solution.    The experimental curve, however, deviates markedly from the calculated values.    It does not lie between the calculated maximum and the calculated minimum values; it is considerably below the calculated minimum curve.    Moreover the specific conductance *falls to values below that of the 0.005 N sodium citrate solution itself.*    After about 1½ equivalents of sodium citrate have been added, the direction of the curve changes; the specific conductance then increases with further additions of sodium citrate.

*Experiment 5.    Titration of 0.005 N Ca Solutions with Sodium Citrate.*

The peculiar behavior observed in Experiment 4 was investigated further.   Another aliquot of the $CaCl_2$ solution prepared

TABLE IV.

*Titration of $CaCl_2$ with Na Citrate.*

Initial solution, 20.0 cc. of 0.005 N $CaCl_2$.

| 0.005 Na citrate added. | Total volume. | Specific conductance of mixture. |
|---|---|---|
| cc | cc. | mhos $\times 10^4$ |
| 0 0 | 20.0 | 9.10 |
| 5.0 | 25 0 | 8.03 |
| 10 0 | 30.0 | 7.10 |
| 15 0 | 35.0 | 6 56 |
| 17 0 | 37 0 | 6.23 |
| 19 0 | 39 0 | 6.14 |
| 20 0 | 40 0 | 6.07 |
| 21.0 | 41 0 | 5.94 |
| 22 0 | 42 0 | 5 86 |
| 23 0 | 43.0 | 5.82 |
| 24 0 | 44.0 | 5.83 |
| 25 0 | 45.0 | 5.78 |
| 26 0 | 46 0 | 5 68 |
| 27 0 | 47.0 | 5.64 |
| 28.0 | 48.0 | 5.60 |
| 29.0 | 49.0 | 5.69 |
| 30.0 | 50.0 | 5.59 |
| 31.0 | 51.0 | 5.57 |
| 32.0 | 52.0 | 5.58 |
| 33.0 | 53.0 | 5.57 |
| 35.0 | 55.0 | 5.59 |
| 38.0 | 58.0 | 5.60 |
| 41.0 | 61.0 | 5.66 |
| 45.0 | 65.0 | 5.71 |
| 50 0 | 70 0 | 5.93 |

Specific conductance of the 0.005 N Na citrate solution    6.80 $\times$ 10⁻ mhos.

from $CaCO_3$ was diluted to 0.005 N without adjustment of the pH. This acid solution of 0.005 N $CaCl_2$ was titrated with 0.005 N sodium citrate for the sake of comparison with the solution with
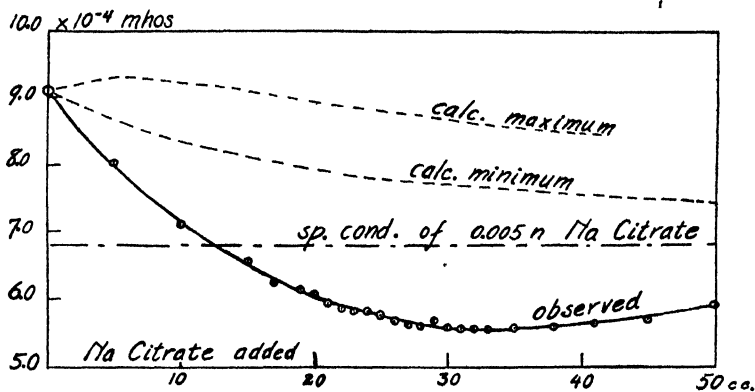
FIG. 4. Titration of 0.005 N CaCl₂ with 0 005 N Na citrate.
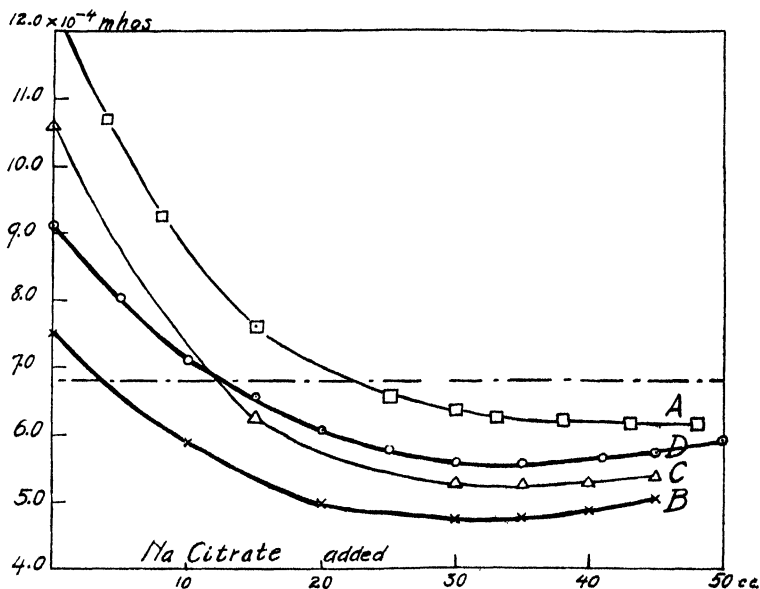


FIG. 5. Titration of 0.005 N Ca solutions with 0.005 N Na citrate.   Curve A indicates Ca(OH)₂; Curve B, CaCl₂ from Ca(OH)₂, pH 7.4; Curve C, CaCl₂ from CaCO₃, acid solution; Curve D, CaCl₂ from CaCO₃, pH 7.4.

adjusted pH. The results of the titration are represented by Curve C in Fig. 5. The experimental curve from the preceding experiment is given again in Fig. 5 for the sake of comparison; it is here labelled Curve D. It is seen that both curves fall to values below the specific conductance of the 0.005 N sodium citrate solution itself, which is represented by the dot and dash line.

The question arose as to whether the method of preparing these calcium solutions was responsible for this unique behavior. To settle this point a stock solution of $Ca(OH)_2$ was prepared from calcium oxalate as described at the beginning of this paper. One aliquot was diluted to 0.005 N $Ca(OH)_2$, and was titrated with the sodium citrate solution. The results are represented by Curve A in Fig. 5. It is seen that this solution behaves in a fashion similar to the acid and neutral solutions of $CaCl_2$.

Another aliquot of the $Ca(OH)_2$ solution was made up to volume after addition of enough HCl to give a pH of 7.4. This solution had the same concentration of calcium as the others, i.e. 0.005 N. The titration values for this solution are represented by Curve B in Fig. 5. It is seen that Curve B has almost the same shape as Curve D. These two solutions of 0.005 N $CaCl_2$, both with pH 7.4, give almost identical curves although one was prepared from $Ca(O)_i$ and the other from calcium oxide. The chief difference is the displacement along the Y axis. Curve D is higher than Curve B because of the presence of NaCl resulting from the neutralization of the excess HCl with NaOH when the pH was adjusted to 7.4

*Experiment 6. Titration of Sodium Citrate with Calcium Chloride.*

The same type of curve is obtained whether the $CaCl_2$ is titrated with sodium citrate, or *vice versa*. In the titration of 20.0 cc. of 0.005 N sodium citrate with 0.005 N $CaCl_2$ (Table V) it will be seen that as the $CaCl_2$ solution is added, the specific conductance decreases to a minimum value, after which the specific conductance increases; here again there is a point of inflection in the experimental curve.

*Dilution Titration Curves.*

All of the dilution titration experiments are given graphically in Fig. 6.

TABLE V.

*Titration of Na Citrate with CaCl₂ (pH Not Adjusted; Acid with HCl).*
Initial solution, 20 0 cc. of 0 005 N Na citrate.

| 0 005 N CaCl₂ added | Total volume | Specific conductance of mixture |
|---|---|---|
| *cc* | *cc* | *mhos × 10⁴* |
| 0 0 | 20 0 | 6 98 |
| 5 0 | 25 0 | 5 79 |
| 10 0 | 30 0 | 5 17 |
| 12 0 | 32 0 | 5 10 |
| 15 0 | 35 0 | 5 18 |
| 17 0 | 37 0 | 5 30 |
| 19 0 | 39 0 | 5 41 |
| 23 0 | 43 0 | 5 74 |
| 30 0 | 50 0 | 6 20 |
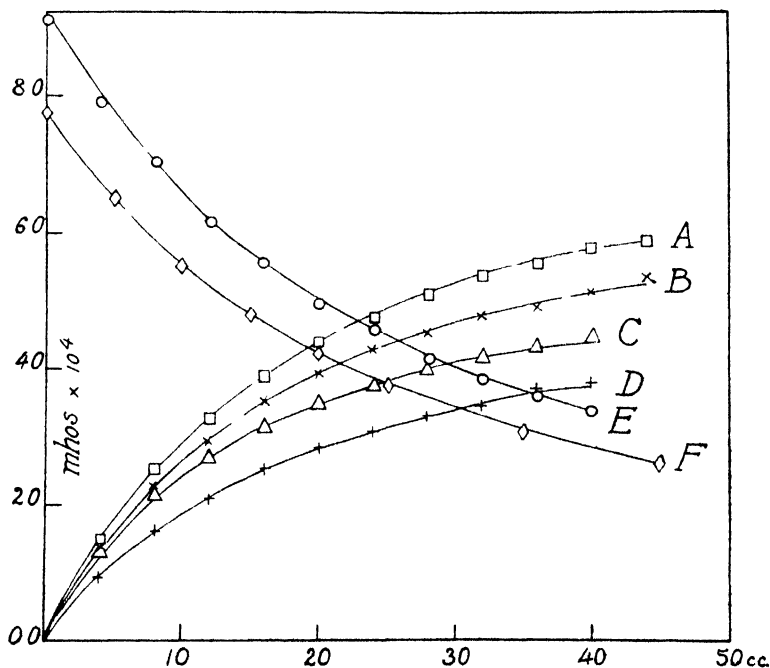


FIG 6  Dilution curves    Curve A indicates NaCl; Curve B, Na citrate.
Curve C, Na acetate; Curve D, Na lactate; Curve E, CaCl₂ from CaCO₃;
Curve F, CaCl₂ from CaO.

The results obtained at 38° are in agreement with those which we previously reported for 25°.   In the studies at 25° it was found that titration of $CaCl_2$ with sodium citrate gave abnormal results, while titration of NaCl with sodium citrate gave normal results. The abnormal behavior of $CaCl_2$, when sodium citrate is added to it, is therefore apparently not due to the chloride ion but to the calcium ion.

At 38° titration of $CaCl_2$ with sodium chloride, sodium acetate, and sodium lactate gave normal results, while titration with sodium citrate gave abnormal results.   Thus the peculiar effect of sodium citrate on calcium ion apparently is due not to the sodium ion but to the citrate ion.

There thus appears to be an ionic reaction of some sort between calcium ions and citrate ions.   The type of curve obtained here with calcium and citrate ions is that obtained in conductivity titrations when ions are removed from solution in the form of a soluble, slightly ionized compound, or in the form of a precipitate (4).   Since no precipitate is obtained, these findings are taken as direct evidence for the formation of a slightly ionized, soluble calcium citrate complex.

The ratio of calcium to citrate in this complex may be found by determining the end-point of the titration, as described by Kolthoff and others.   This point is determined by carrying the titration for some distance past the point of inflection and by extrapolation of the two approximately linear portions of the curve to their point of intersection.   An accurate determination of this end-point would require that the experimental curve in Fig. 4 be carried further until an approximately straight line is obtained after the point of inflection is passed.   An estimate from the curve in Fig. 4 would place the end-point at about 20 cc.; *i.e.*, the reaction appears to involve approximately equivalent quantities of calcium and citrate ions.

In the experiments reported here the solutions were frequently exposed to the air of the laboratory during the titrations.   The upper part of the Pyrex cell projected out of the water bath; there was at times some condensation, on the inside of this projecting portion of the cell, of the water vapor rising from the warm solution.   The error from these and other causes is estimated to be

about 3 per cent.   The conductance of different preparations of the
same solution differed somewhat as is seen from the initial values
for 0.005 N CaCl₂ (from CaCO₃) given in Tables I to IV.   These
differences appear to be due to a slight variation in the concentra-
tion of NaCl; the amounts of NaOH required for the adjustment
of the pH of different aliquots of the stock CaCl₂ solution were not
always identical.   Although the specific conductance of the initial
solution of 0.005 N CaCl₂ varied somewhat from one preparation
to another, the titration curves for duplicate experiments were the
same; they differed only in being placed slightly higher or lower
with respect to the Y axis.

The effect of dilution on the specific conductance of each salt
was ascertained by means of dilution titrations.   In making the
calculations, the conductance of each original solution was "cor-
rected" for each different mixture.   This is in no sense a real
correction; it is only a convenient calculation in an empirical
method of obtaining the approximate upper limit of the specific
conductances of the various mixtures.   Quotation marks have
been used throughout this paper to avoid giving the impression
that the "corrected" values are any more correct than the ex-
perimentally determined values.

There are a number of questions in regard to the state of the
serum calcium upon which light may be thrown by the applica-
tion of similar conductivity titrations.   For example, it may be
possible in this way to determine whether calcium ions form a
soluble, unionized complex with the parathyroid hormone, or
with the various proteins present in serum.   Such studies are in
progress in this laboratory.

The theory of complete dissociation of strong electrolytes has
been quite successful during the last six years in providing a
theoretical basis for a considerable number of experimental ob-
servations.   However, this does not necessarily mean that all
salts are completely dissociated.   Thus McBain and Van Ryssel-
berge (5) obtained data on the migration of calcium and magnesium
ions which show that these cations may combine with anions to
form unionized complexes or complex anions.   Stewart and Perci-
val (6) have recently stated that they have confirmed, by means of
conductivity measurements of calcium citrate solutions, the
commonly held theory that calcium citrate is almost undissociated;
however, they gave no data or details.

The results reported here confirm the theory held by a number of investigators to account for the behavior of biological systems following the addition of sodium citrate; it is generally supposed that the citrate ions combine with the calcium ions to form unionized calcium citrate.[3]  Shelling and Maslow (10) recently reported that intravenous injection of sodium lactate or sodium acetate into rabbits does not produce convulsions; the serum calcium of such rabbits was found to be about 50 per cent ultrafiltrable, the same as the serum calcium of untreated rabbits.  Similar injection of sodium citrate, however, produced convulsions and death; the serum calcium of these rabbits was found to be nearly 100 per cent ultrafiltrable.  Similar results were obtained when the sodium citrate was added to serum *in vitro; i.e.*, almost all of the calcium passed into the ultrafiltrate.  These experiments were performed on sera from sheep, cows, and humans.  These authors stated, "Our ultrafiltration experiments and the observations of Shear and Kramér would seem to support Sabatini's hypothesis; namely, that the citrate ions combine with calcium to form an unionized compound and thus rob the organism of its active calcium necessary for muscular and nervous stimulation and inhibition."

## SUMMARY.

1. Sodium chloride, sodium acetate, and sodium lactate give normal conductivity titration curves with $CaCl_2$ at 38°.

2. Sodium citrate gives abnormal curves with solutions of calcium ions at 38°.  The same type of curve is obtained regardless of whether the calcium solution is acid, neutral, or alkaline in reaction.

3. These results are further evidence for the binding of calcium ions by citrate ions in some kind of soluble slightly ionized complex.

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[3] Salant and Hecht (7) and Salant and Swanson (8) have, however, expressed a dissenting opinion.  The effect of citrate on calcium ions may be obscured in perfusion experiments by other factors, as was shown in the interesting experiments recently reported by Clark, Percival, and Stewart (9).

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# THE CALCIUM CONTENT OF MUSCULAR TISSUE DURING PARATHYROID TETANY.

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*(From the Institute of Neurology, Northwestern University Medical School, Chicago.)*

Excepting the observations of Loughridge (1) we have been unable to find data on the calcium content of striped muscle during parathyroid tetany. He found much less calcium in dog muscle during tetany (two animals) than in the muscles after recovery from tetany through treatment with calcium chloride or parathyroid extract (three animals). Katz (2) found 0.0685 parts of calcium per kilo of normal dog muscle. Behrendt (3) stated that the calcium content of muscles remains unchanged during parathyroid tetany in dogs, but gave no data. MacCallum and Voegtlin (4) found that the calcium content of brain tissue was reduced and assumed that it was lower in other tissues. Cooke (5) found slightly more calcium in the brains of dogs dying with tetany than in normals.

On account of the small amount of existing data we thought it desirable to determine calcium in the muscles of normal and of parathyroidectomized dogs either to confirm previous reports or add new findings.

## EXPERIMENTAL.

Our series comprised nine parathyroidectomized dogs and eight normal ones. The parathyroidectomized animals were sacrificed from 3 to 8 days after operation. Mixtures of gluteal and quadriceps femoris muscles were used for analysis. The tissue was finely ground in a meat chopper and, after mixing, was divided into 20 gm. samples. Duplicate samples were always taken and in several determinations triplicates were used. The tissue was

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extracted for about 48 hours (with frequent shaking) in 200 cc. of 5 per cent trichloroacetic acid. The fluid was filtered from the residue, the volume noted, 10 cc. of concentrated nitric acid added, and the sample evaporated to about 5 cc. This was transferred to tapered centrifuge tubes, neutralized with concentrated ammonia water to approximately pH 6.0, and 1 cc. of saturated ammonium oxalate added for every 5 cc. of the neutralized fluid. The tubes were allowed to stand overnight, then centrifugalized, and the precipitate washed and titrated as in the Kramer-Tisdall method for serum calcium. Determinations on standard solutions of calcium showed that recoveries of about 95 per cent could be expected. The calcium obtained from the muscle by the above

TABLE I

*Calcium Content in Mg. per 100 Gm of Mixed Muscles Expressed as Average of Duplicate or Triplicate Determinations*

P, parathyroidectomized; N, normal.

| Dog No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | Maximum | Minimum | Average. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P | 9.5 | 8.3 | 10.5 | 5.2 | 7.0 | 8.9 | 7.5 | 8.0 | 8.9* | 10.5 | 5.2 | 8.2 |
| N | 5.8 | 4.8 | 9.4 | 7.6 | 10.5 | 8.0 | 8.9 | 6.7 | | 10.5 | 4.8 | 7.7 |

* The dog developed a respiratory infection and was killed on the 4th day after operation; did not develop tetany.

Calcium in whole blood (trichloroacetic acid filtrate), Dogs P1, 2.3 mg ; P2, 3 6 mg.; P6, 4 1 mg.; P9, 1.4 mg.; Dog N1, 5.8 mg.

Severe tetany was present in Dogs P1, P2, and P8; moderate to mild tetany in Dogs P3, P4, P5, P6, and P7.

procedure represented the amount which is acid-extractable and comes to equilibrium between tissue and fluid during the extraction process. The amount of calcium obtained multiplied by the quotient:

$$\frac{\text{Volume of muscle + extraction fluid}}{\text{Filtrate obtained}}$$

and converted into mg. per 100 gm. of tissue gave the results expressed in Table I.

Our data show a slightly higher average for the tetany series than for the normal animals but we believe that with a greater number of animals the difference would disappear. There was no

consistent variation between the two groups, although the individual variation in both was very great. The relatively large variation seen in different animals suggests that there may be sources of calcium in muscular tissue other than the calcium supposed to be present in muscular elements proper. If it is chiefly an integral part of the latter and has a definite function, the variations are remarkable. It seems noteworthy that Urano (6) found no calcium in *Press-saft* of muscles of frogs. Knowledge of the site of calcium in muscular tissue might lead to a better understanding of its function.

The findings agree with the report by Behrendt (3) but not with the conclusions drawn by Loughridge (1). However, our work is not directly comparable with his since we had no treated animals. One of our parathyroidectomized animals showed as low calcium as his untreated ones. Further work will be necessary to determine whether muscles will take up calcium when its salts are introduced parenterally or animals are treated with parathyroid extract.

A myogenic origin of tetania parathyreopriva is further negated by these findings.

### SUMMARY.

The calcium content of striped muscle of nine parathyroidectomized dogs was found to lie in the same range as that of eight normal animals.

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# MUSCLE PHOSPHORUS.

## III. THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS COMPOUNDS DURING PARATHYROID TETANY.

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Tetany produced by removal of the parathyroid glands is characterized by periods of intense muscular activity, and since the compounds of phosphorus participate in muscular activity, it seemed desirable to determine what changes might occur. If changes were found they could then be compared with those which occurred during stimulation of the muscles of normal animals.

The partition of phosphorus compounds in muscular tissue as given here is based upon the principles discussed in Paper II of this series (2). Inorganic phosphate (ortho-), phosphocreatine, pyrophosphate,[1] and the remaining compounds to include the total were estimated.

We were unable to find previous reports of work on muscle phosphorus during parathyroid tetany except one in 1926 by Loughridge (7). His experiments were based on the determination of A and B values according to Embden's procedure, and the changes found were considered to be secondary to fatigue. Biedl's monograph of 1922 (1) (pp. 267–320) contained with reference to phosphorus metabolism only the early observation of Greenwald regarding a diminished excretion of phosphorus after parathyroidectomy. In more recent work Greenwald and Gross (5) have

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[1] The fraction designated here as pyrophosphate is based upon the findings of Lohmann (6). It corresponds to about 80 per cent of the phosphorus-containing material formerly designated by Embden as lactacidogen and is the same as Lactacidogen I in Paper II of this series.

shown that in thyroparathyroidectomized dogs there is a decreased urinary excretion of phosphorus but that the amount in the feces remains unchanged.    After administration of parathyroid extract to normal dogs the urinary phosphorus was increased.

Our series consisted of ten dogs, five of which were thyroparathyroidectomized and five were normal.    The results obtained were negative with reference to any specific change in muscle phosphorus, either total content or change in the partitions studied.

### EXPERIMENTAL.

The gastrocnemii were used for analysis and were removed from he animal and prepared for analysis in the manner described in a preceding paper (9).    Amytal anesthesia was used.    In Dogs P1 and P2 the muscles were frozen during tetanic spasms which persisted for about $\frac{1}{2}$ hour after the induction of anesthesia.    In the remaining animals with tetany, the muscular activity disappeared when anesthesia was established.

The values for the phosphorus fractions were obtained by the procedures previously outlined (2, 9) except that the value (previously called H) for the sum of inorganic, phosphocreatine, and pyrophosphate phosphorus was obtained by hydrolyzing in a boiling water bath an aliquot of filtrate for 15 minutes with 1 N hydrochloric acid instead of 1 hour with sulfuric acid of the same concentration.    This variation in method brings our data into agreement with the determination of the pyrophosphate fraction according to the findings of Lohmann (6).    In the preceding paper, Davenport and Sacks (2) reported that there appeared to be no free pyrophosphate in a trichloroacetic acid filtrate of fresh muscle. The conclusion was based on the failure of this filtrate to develop a characteristic tint of blue color after standing for 2 hours with Fiske's phosphate reagents.    Since then, the senior author has partially isolated the pyrophosphate fraction from muscle filtrates, by means of Lohmann's technique, as a barium salt and found that the material gave the characteristic color in concentrations comparable to that of pure pyrophosphate.    There is therefore no disagreement between our work and Lohmann's regarding the identity of the fraction formerly called lactacidogen.    It would seem that the pyrophosphate is either loosely bound in the un-

TABLE I

*Distribution of Acid-Soluble Phosphorus in Gastrocnemii of Parathyroidectomized (Dogs P1 to P5) and Normal Dogs (Dogs N1 to N5).*

| Muscle No | | Days after operation | Acid-soluble P,* mg per gm | | | | | Remarks. | N |
|---|---|---|---|---|---|---|---|---|---|
| | | | I | Pc | Py | R | T | | |
| | | | | | | | | | *mg. per gm.* |
| Parathyroidectomized. | | | | | | | | | |
| P1 | R | 3 | 18 | 38 | 54 | 31 | 141 | Nerve intact. | 31.5 |
| | L | | 19 | 31 | 46 | 29 | 125 | Denervated ½ hr. | Lost. |
| P2 | R | 6 | 22 | 48 | 46 | 33 | 149 | ⎫ | 32.3 |
| | L | | 25 | 46 | 45 | 34 | 150 | ⎪ | 32 4 |
| P3 | R | 5 | 21 | 39 | 35 | 35 | 130 | ⎬ Nerves intact. | 29.0 |
| | L | | 21 | 39 | 36 | 31 | 127 | ⎭ | 29 5 |
| P4 | R | 5 | 31 | 33 | 36 | 39 | 139 | Unstimulated. | 30.9 |
| | L | | 25 | 45 | 37 | 38 | 145 | Stimulated 4 hrs † | 32.2 |
| P5 | R | 2 | 21 | 45 | 35 | 31 | 132 | Unstimulated | 32.6 |
| | L | | 37 | 10 | 45 | 35 | 127 | Tetanized 4 min | 33.0 |
| Normal. | | | | | | | | | |
| N1 | R | | 28 | 42 | 34 | 31 | 135 | Unstimulated. | 29.6 |
| | L | | 29 | 39 | 34 | 32 | 134 | " | 29.6 |
| N2 | R | | 20 | 44 | 46 | 29 | 139 | Unstimulated. | 31.4 |
| | L | | 18 | 42 | 42 | 25 | 127 | Stimulated 2½ hrs.† | 30.6 |
| N3 | R | | 23 | 39 | 48 | 34 | 144 | Unstimulated | Not |
| | L | | 28 | 28 | 55 | 35 | 136 | Tetanized 3 min. | done. |
| N4 | R | | 31 | 41 | 42 | 34 | 148 | Unstimulated | 32 2 |
| | L | | 35 | 28 | 41 | 32 | 136 | Tetanized 3 min. | 31.9 |
| N5 | R | | 19‡ | 49 | 38 | 36 | 142 | Unstimulated. | 29.0 |
| | L | | 22‡ | 47 | 40 | 33 | 142 | " | 29.3 |

\* I inorganic, Pc phosphocreatine, Py pyrophosphate fraction, R remainder (relatively acid-stable), T total (sum of I, Pc, Py, and R).

† Interrupted tetanus; ‡ second on and ⅛ second off through the motor nerve and about two-thirds maximal in strength.

‡ By precipitation as Ca salt.

treated filtrate and the combination is broken by barium precipitation, or there is some substance present which interferes with the color reaction in untreated filtrate and this substance is removed in purifying the barium salt.

The demonstration of the fraction previously referred to as Lactacidogen II (2) has not proved as satisfactory in the dog as in the rabbit, hence we have omitted the B value in the present work. Several incubations were made on muscles from both normal and parathyroidectomized animals but the B value showed no characteristic variations in either resting or stimulated muscle.

Table I shows several features which seem worth noting. In previous experiments with frogs (3) we found that the total phosphorus content of gastrocnemii was apparently increased by the intraperitoneal injection of massive doses of neutral phosphate solution.   This increase was not due to absorption of phosphate because the protein nitrogen of the tissue increased per unit weight proportionately to the phosphorus, therefore the phosphorus increase was due to dehydration.   During parathyroid tetany, dogs lose weight rapidly and in order to ascertain whether dehydration of their muscles occurred, we determined the nitrogen in the protein residues after the nitrogenous and other extractives were removed.   The nitrogen data show that dehydration of the muscles did not occur, for the values vary from 29 to 33 mg. of protein N per gm. of fresh tissue, and these values are essentially the normal range for adult dogs.   Likewise the total phosphorus content is normal    High phosphorus was usually coincident with high nitrogen and *vice versa*.

Muscles which were stimulated tetanically through the nerve showed less total phosphorus than unstimulated muscles, but no change in residue nitrogen (Dogs P5 and N4), hence hydration of the tissue did not appear to be a factor in reduction of total phosphorus.

This change seems to be explained by vasodilatation.   Blood filtrate contains only about one-fourth as much phosphorus as muscle filtrate and the decrease observed could readily have been due to the diluting effect of blood in the muscular tissue.   This effect was not seen in Muscle P4L but was present in Muscle N2L.   Both of these muscles were stimulated indirectly with brief tetanic shocks at the rate of $\frac{1}{8}$ second on and $\frac{1}{8}$ off for 4 and

for $2\frac{1}{2}$ hours respectively. Such intermittent stimulation produced practically no change in the distribution of phosphorus in either the parathyroidectomized or normal animal.

Muscle P1L shows a decreased total phosphorus content as a result of cutting its nerve. The explanation is most likely that vasodilatation occurred as in the stimulated muscles.

It will be noted that the inorganic phosphate content varies considerably—from 18 to 31 mg. per cent in supposedly resting muscles—but that the variation has no relationship to parathyroidectomy. The determination of the inorganic phosphate by precipitation with magnesia mixture (9) has been appropriately criticized by Fiske and Subbarow and we welcome their improved method of precipitation by calcium (4). Direct comparisons of phosphate determinations from fresh muscle filtrates have shown that the calcium precipitation method gives results 15 to 30 per cent higher than those obtained by magnesia mixture. We have not found such differences in tests on standard solutions of phosphate of the same concentration as that in the muscle filtrate. The recovery by magnesia mixture varied from 92 to 97 per cent, while that by calcium was quantitative as nearly as a colorimetric method would show. The reason for the greater divergence between the two procedures on muscle filtrate than on standard solutions of similar concentration may be explained either by the failure of magnesia mixture to effect as complete a precipitation in the filtrate (as suggested by Fiske and Subbarow) or by the precipitation of color-intensifying substances by calcium. The latter seems rather improbable, hence we are inclined to believe at present that the precipitation of phosphate as the calcium salt is the method of choice. The data on inorganic phosphate (I values) reported here were obtained, with the exception of those for Dog N5, by magnesia mixture precipitation. An arbitrary addition of 10 per cent (based on the comparative study of the two methods) has been made on the other I values. They may still be somewhat too low, provided one adopts the value obtained by calcium precipitation as 100 per cent.

Our findings give no indication that parathyroid tetany has a myogenic origin. This is not in agreement with the opinions of Martin (8). Likewise no support to a theory that dehydration of muscle might play a rôle, as we were inclined to believe at the

beginning of the work, was obtained. The physiological reactions in muscle which involve the phosphorus compounds studied are evidently so reversible that no static change was demonstrable.

### SUMMARY.

1. No changes in acid-soluble phosphorus content nor in the partitions of phosphorus compounds were found in the gastrocnemii of parathyroidectomized dogs.

2. Dehydration of these muscles was not demonstrable.

3. Changes in phosphate metabolism during parathyroid tetany involve a source of phosphorus other than that of striped muscle.

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# THE EFFECT OF AN EXCLUSIVE MEAT DIET LASTING ONE YEAR ON THE CARBOHYDRATE TOLERANCE OF TWO NORMAL MEN.*

By EDWARD TOLSTOI.

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During the past year exhaustive studies were made of the metabolism of two normal men whose diet consisted for 1 year exclusively of lean and fat meat. This was essentially a high fat, low carbohydrate diet. The tolerance of these men to carbohydrate was tested by the glucose meal. As far as can be ascertained, no experiments have been reported where subjects lived on a similar diet for 1 year. This communication presents such experiments.

## EXPERIMENTAL.

The subjects were healthy adult men whose diet consisted solely of lean and fat meat. This was eaten either cooked or raw. The protein of the diet averaged 120 gm. with a caloric intake of 2600 to 3000 calories. Other data and general plan of the experiment will be published by McClellan and Du Bois (1). Two glucose tolerance tests were carried out on each of the two subjects, K. A. and V. S.; one test immediately after the meat-fat diet was discontinued, the other 2 or more weeks after the general diet had been resumed. Some 8 or 9 hours after the first glucose tolerance test one of the men (K. A.) developed a pneumonia from which he recovered and reported in good condition for his second test 4 weeks later. Subject V. S. was in good health and showed no abnormalities either before or after any of the tests. Each man

received 100 gm. of glucose in 230 cc. of water. About 20 cc. of
orange juice were added for flavoring. This solution was given
10 to 12 hours after the last meal. Blood samples were obtained
from a vein at the elbow before the test meal, and $\frac{1}{2}$, 1, 2, and 3
hours after it. The blood was discharged into a flask containing
about 2 mg. of potassium oxalate for each cc. of blood. A portion
was at once precipitated and the sugar determined by the method

TABLE I.

*Reducing Bodies.*

| Subject | Time | Blood. | Urine. | Remarks. |
|---------|------|--------|--------|----------|
| | *hrs* | *mg. per cent* | | |
| K. A. | Fasting. | . 110 | 0 | After diet of lean and |
| | $\frac{1}{2}$ | 235 | $++++$ | fat meat for 1 yr. |
| | 1 | 235 | $++++$ | |
| | 2 | 250 | $++++$ | |
| | 3 | 200 | $++++$ | |
| | Fasting. | 87 | 0 | After general diet for |
| | $\frac{1}{2}$ | 166 | 0 | 4 wks. |
| | 1 | 153 | 0 | |
| | 2 | 97 | 0 | |
| | 3 | 84 | 0 | |
| V. S. | Fasting. | 105 | 0 | After diet of lean and |
| | $\frac{1}{2}$ | 200 | 0 | fat meat for 1 yr. |
| | 1 | 210 | 0 | |
| | 2 | 182 | 0 | |
| | 3 | 117 | 0 | |
| | Fasting | 105 | 0 | After general diet for |
| | $\frac{1}{2}$ | 143 | 0 | 2 wks. |
| | 1 | 122 | 0 | |
| | 2 | 116 | 0 | |
| | 3 | 98 | 0 | |

of Folin and Wu (2). The urine of each subject was examined for
reducing substances whenever samples were obtained during the
course of the test.

The results obtained by these procedures are presented in
Table I and Charts 1 and 2. Both show that a marked rise in the
blood sugar occurred after the test meal following the previous high
fat, low carbohydrate diet. The curve of K. A. not only in-

creased in height, but the hyperglycemia was prolonged also. He presented a glycosuria. V. S. had only an increase in the height of the curve. His urine was sugar-free. After a mixed diet for the periods mentioned above, both men reacted normally to the glucose tolerance tests, the height and the duration of the blood sugar curve being well within normal limits, and the urine sugar-free.

Subject K.A.
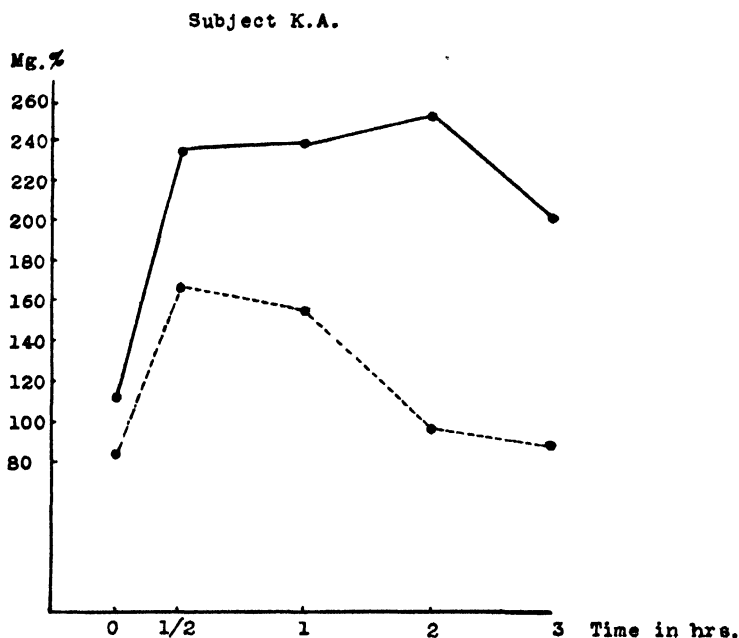


CHART 1. Blood sugar curves after glucose tolerance tests. Solid line indicates results after a diet of lean and fat meat for 1 year, dotted line after a general diet.

### DISCUSSION.

The above results are not new. They were emphasized by Odin (3), Malmros (4), Stenstrom (5), Staub (6), Kageura (7), Greenwald, Gross, and Samet (8) and others. It was believed that during a period of a low carbohydrate, high fat diet, the need for insulin was diminished, with a resulting decrease in its production. Then, upon administration of a large quantity of carbohydrate to a subject subsisting on such a diet, the carbohydrate

mechanism is heavily taxed. The production of insulin cannot keep up with the demand, the result being a hyperglycemia and often a glycosuria as well. The extensive and excellent work of Malmros (4) also supports the above view. He worked with normal human beings who had glucose tolerance tests after general diets as well as after high fat, low protein, and low carbohydrate mixtures. The duration of such diets varied from 1 to 23 days.
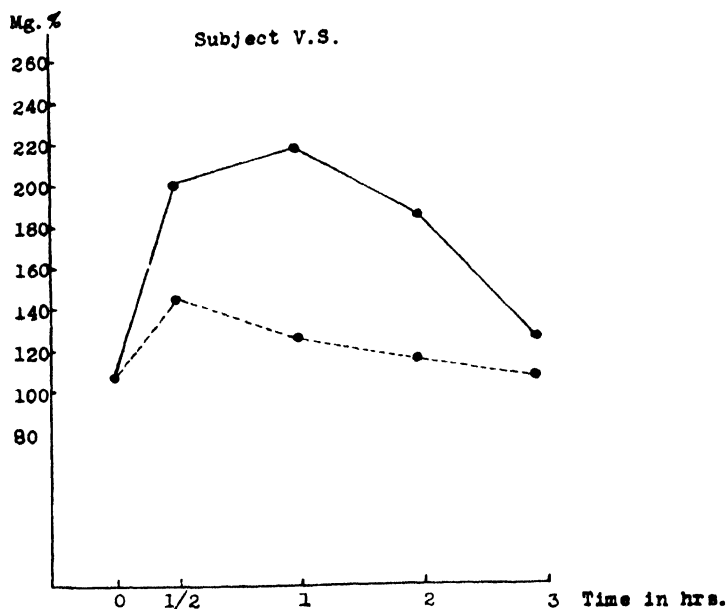


CHART 2    Blood sugar curves after glucose tolerance tests. Solid line indicates results after a diet of lean and fat meat for 1 year, dotted line after a general diet.

In every case a lowering of the tolerance to carbohydrate was noted irrespective of the duration of the preceding diet. Greenwald, Gross, and Samet (8) were of the same opinion. They believed that a diet consisting chiefly of protein does not lower the tolerance for glucose as much as a diet consisting principally of fat. This inference might be used to explain the difference between the results of Heinbecker (9) and the ones presented in this paper.

Heinbecker studied the tolerance of Eskimos to carbohydrate. His subjects, by necessity, lived on a practically exclusive meat diet for years, before their carbohydrate tolerance tests were made. In spite of the fact that their diets were low in carbohydrate, the results of the tests indicated that they assimilated carbohydrate well. The blood sugar curves were within the normal range and the urine remained free of sugar. Is it possible that Heinbecker's subjects derived sufficient carbohydrate-forming substance from the protein in their diet to keep the insulin producing mechanism sufficiently stimulated to handle large quantities of carbohydrate? His Eskimos consumed about 280 gm. of protein, 135 gm. of fat, and 54 gm. of carbohydrate of which more than half is obtained from the glycogen of the meat.[1] This seems a likely explanation.

If then a low carbohydrate, low protein, and high fat diet lowers the tolerance to carbohydrate in normal human beings, is it reasonable to assume that a high carbohydrate diet will raise the tolerance?

This too has been demonstrated. John (11) performed glucose tolerance tests on two normal subjects to whom he gave 100 gm. of glucose on 5 successive days. And although the peak of the blood sugar curve was 260 mg. on the 1st day, the maximum was only 90 mg. on the 5th day. Traugott's (12) experiments concur with this work. He gave an initial dose of 20 gm. of glucose, obtaining an increase in the height of the blood sugar curve. When, an hour later, after the first 20 gm. additional amounts of glucose were given in various quantities, from 20 to 100 gm., no hyperglycemia was noted. Apparently the initial stimulus of 20 gm. evoked sufficient amounts of sugar-metabolizing hormone, to take care of the additional quantities of glucose given later.

Such experiments and the ones presented above lead to the belief that in normal human beings, the quantity of insulin produced is dependent upon the amount of carbohydrate ingested irrespective of whether this foodstuff is administered as such or derived from the protein fraction of the diet.

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[1] These figures are based on the analytical data of Krogh and Krogh (10), and are acceptable as Heinbecker points out that the statements regarding the Greenland Polar Eskimo, the peoples studied by the Kroghs apply to Baffin Island Eskimos. the group studied by him.

The question of the effect of ketosis on the glucose tolerance has not been discussed as that phase of the subject has been presented fully and clearly by Malmros (4).

## SUMMARY AND CONCLUSIONS.

Two normal men were given glucose tolerance tests after both had lived for 1 year on lean and fat meat exclusively, (protein 120 gm., 2600 to 3000 calories) and later after a general diet. Following the meat diet there was a diminution of the tolerance to glucose as demonstrated by the blood sugar curve of both men and a glycosuria of one man. After 2 to 4 weeks of a general diet the blood sugar curve presented no abnormalities and the urine was sugar-free. The explanation of this phenomenon is that the normal carbohydrate mechanism needs daily stimulation for good function. Should that stimulus be lacking as is the case in low carbohydrate, high fat diets, and in prolonged fasting, it is temporarily incapable of handling large quantities of carbohydrate. In normal human beings this mechanism recovers fully after a general diet. These data are of practical importance in pointing out a probable fallacy in the interpretation of the glucose tolerance test, when the factors here discussed are not considered.

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# THE EFFECT OF AN EXCLUSIVE MEAT DIET ON THE CHEMICAL CONSTITUENTS OF THE BLOOD.*

## BY EDWARD TOLSTOI.

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This paper presents the chemical findings in the blood of two normal men, whose diet for 1 year consisted exclusively of lean and fat meat. While this study was in progress Heinbecker (1) published the results of similar experiments. He studied certain phases of the metabolism of the Baffin Island Eskimos and reported some chemical analyses of the blood. The constituents determined were within normal limits. Because of the lack of facilities where the experiments were conducted Heinbecker found it necessary to bring the filtrates of the blood to his laboratory in St. Louis where the work was completed. The blood filtrates contained moulds, and though the values for the non-protein nitrogen were normal the results may be questioned, because of certain changes occurring in the filtrates of blood on standing.

In both experiments the subjects subsisted on meat alone, but the composition of the diet was not the same in each instance. Heinbecker's Eskimos ate about twice as much protein and about half as much fat as the men studied by us. Our subjects consumed about 120 to 130 gm. of protein and enough fat to make a total intake of 2600 to 3000 calories per day. A full description of the experiment of which this is a part will be published by McClellan and Du Bois (2).

At the outset it was difficult to decide which chemical constituents of the blood were to be studied. The possibility of renal damage suggested, of course, the study of some of the non-protein

Meat Diet Effect on Blood

nitrogenous substances. The traditional association of meat with uric acid metabolism made the study of the uric acid desirable. As the plasma showed a milkiness soon after the beginning of the experiments, the blood cholesterol was observed. Only a few analyses of the calcium and phosphorus were made. The

TABLE I.

*Blood Figures of Subject K. A. Expressed in Mg. per 100 Cc.*

| Date. | Non-protein N. | Urea | Uric acid. | Creatinine. | Sugar. | Serum Ca. | Plasma P. | NaCl | R.B.C. | Plasma. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *1928* | | | | | | | | | *per cent* | *per cent* | |
| Jan. 21 | 40 | 21 | 3.7 | 1.58 | 107 | | 3.5 | 450 | | | Before meat |
| " 25 | 40 | | | | | | | | | | diet. |
| Feb. 1 | 43 | | 3.5 | 1.70 | 92 | | 3.6 | 455 | | | |
| Mar. 7 | 39 | 19 | | | 100 | | | | 43.5 | 56.5 | |
| " 23 | 41 | 22 | 4.0 | 1.66 | 95 | 9.5 | 3.4 | 455 | | | |
| Apr. 13 | 42 | 20 | 3.9 | 1.70 | 93 | 9.8 | | | 40 | 60 | |
| " 23 | 40 | | 4.5 | 1.82 | 111 | 9.9 | 3.9 | | 43.2 | 56.8 | |
| May 28 | 40 | 19 | 5.0 | 1.50 | 105 | 10.0 | | | 45 | 55 | |
| June 25 | 41 | | 5.3 | 1.49 | 91 | 9.6 | | 460 | 45 | 55 | |
| July 26 | 41 | | 4.7 | 1.58 | 105 | | 3.8 | | | | |
| Aug. 25 | No analysis made, as subject had a pharyngitis. | | | | | | | | | | |
| Sept. 25 | 33 | 18 | 4.3 | 1.49 | 96 | 10.0 | | | | | |
| Oct. 25 | 38 | | 4.4 | | 100 | | 3.3 | | | | |
| Nov. 26 | 39 | 20 | 3.0 | 1.50 | 105 | 10.2 | 3.9 | | 47 | 53 | |
| *1929* | | | | | | | | | | | |
| Jan. 9 | 41 | | 3.2 | 1.58 | 105 | 10.0 | 3.7 | 455 | 40 | 60 | |
| " 24 | 42 | 20 | | | 83.5 | Fasted 20 hrs. | | | | | |
| Feb. 15 | 41 | | 3.0 | 1.60 | 110 | | | | | | |
| Mar. 20 | 40 | | | | | | | | | | After general |
| | 41 | | | | | | | | | | diet 4 wks. |
| | 39 | 21 | 3.4 | 1.60 | 87 | 10.0 | 3.7 | 460 | | | |

$CO_2$-combining power was determined because of the ketonuria and its probable effect on the alkali reserve. Analyses of sugar, plasma proteins, and chlorides were also included.

Specimens of blood were obtained before the meat diet was instituted. Thereafter specimens were drawn at monthly intervals and also at the close of the experiment. A portion of the

oxalated blood was at once precipitated. The remainder was centrifuged in graduated tubes for about 20 minutes. After determining the proportion of plasma to cells the former was drawn off and analyzed. About 10 cc. of whole blood were discharged into a test-tube and the serum was used for calcium determination.

TABLE II.

*Data Obtained on Subject K. A.*

Blood figures are expressed in mg. per 100,cc., $CO_2$-combining power in volumes per cent.

| Date. | Plasma proteins. | Albumin. | Globulin. | Cholesterol. | A:G ratio. | CO₂-combining power. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1928* | | | | | | | |
| Jan. 21 | | | | | | 65.5 | Before meat diet. |
| Feb. 1 | | | | | | 59.4 | |
| Mar. 7 | 6.95 | 4.21 | 2.74 | 600 | 1.53 | 60.0 | |
| " 23 | 6.25 | 4.00 | 2.25 | 285 | 1.77 | 67.8 | |
| Apr. 13 | 6.25 | 3.35 | 2.90 | 268 | 1.15 | 60.7 | |
| " 23 | 6.77 | 4.24 | 2.53 | | 1.65 | 66.2 | |
| May 28 | 7.62 | 3.78 | 3.84 | 310 | 0.97 | 52.2 | |
| June 25 | 7.27 | 4.21 | 3.06 | | 1.38 | 60.1 | |
| Sept. 25 | 7.00 | | | | | 61.0 | |
| Nov. 26 | 7.12 | 4.12 | 3 00 | 500 | 1.37 | 56.0 | |
| *1929* | | | | | | | |
| Jan. 9 | 6.85 | | | 400 | | 54.1 | |
| " 24 | | | | 800 | | | After 20 hr. fast. |
| Feb. 15 | 6.74 | | | 415 | | 54.0 | |
| Mar. 20 | 6.98 | | | 200 | | 60.0 | After general diet 4 wks. |

Standard methods were employed. The Folin and Wu (3) system of analysis was used for the non-protein nitrogen, urea, creatinine, and sugar. Uric acid was determined by the method of Benedict (4). Calcium of the serum was analyzed by the procedure of Kramer and Tisdall (5) and the phosphorus of the plasma by the technique of Benedict and Theis (6). The CO₂-combining power of the plasma was determined by the method of Van Slyke and Cullen (7). The technique of Bloor, Pelkan, and

Allen (8) was used for the cholesterol values of the plasma. The total proteins were determined by the macro-Kjeldahl method. 1 cc. of plasma was diluted to 50 cc. with 0.9 per cent sodium chloride and 25 cc. of the diluted plasma were used for each determination, which was made in duplicate. The albumin of the plasma was analyzed by the method of Howe (9). After

TABLE III.

*Blood Figures of Subject V. S. Expressed in Mg. per 100 Cc.*

| Date. | Non-protein N. | Urea. | Uric acid. | Creatinine. | Sugar. | Serum Ca. | Plasma P. | NaCl | R.B.C. | Plasma. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *1928* | | | | | | | | | *per cent* | *per cent* | |
| Feb. 17 | 50 | | | | 117 | | | | | | |
| " 27 | 51 | 28 | 2.7 | 1.57 | 109 | | | | 487 | 39 | 61 | |
| Mar. 2 | 51 | | 3.2 | 1.80 | | | | | | 47 | 53 | |
| " 16 | 53 | 29 | 3.7 | 1.80 | 100 | | | | | | |
| Apr. 20 | 52 | | 4.7 | 1.60 | 115 | 11.0 | 3.4 | 460 | 41 | 59 | |
| May 28 | 50 | 26 | 4.3 | 1.40 | 117 | 11.2 | 3.6 | | 42 | 58 | |
| June 25 | 48 | | 4.7 | 1.43 | 100 | 9.6 | | 470 | | | |
| July 26 | 49 | | 3.4 | 1.50 | 110 | | | | | | |
| Aug. 25 | 46 | 25 | 3.5 | 1.50 | 105 | | 3.9 | | 40 | 60 | |
| Sept. 25 | 45 | | 3.7 | 1.64 | 95 | 10.0 | 3.8 | | | | |
| Oct. 19 | 44 | 24 | 3.7 | 1.52 | 110 | | | 470 | | | |
| Dec. 12 | 50 | 24 | 3.0 | 1.50 | 111 | 10.4 | 3.6 | 475 | 48 | 52 | |
| *1929* | | | | | | | | | | | |
| Jan. 23 | 50 | | 3.5 | 1.61 | 111 | | | | 45 | 55 | |
| Mar. 6 | 48 | 23 | 3.1 | 1.80 | 91 | | | | | | |
| Apr. 4 | 50 | 23 | 3.4 | 1.60 | 105 | 11.0 | 3.4 | 480 | 42 | 58 | |

precipitation of the globulins the nitrogen of the filtrate was determined by the macro-Kjeldahl technique.

Tables I to IV show the results, most of which are self explanatory. From the data it is apparent that the chemical composition of the blood of our subjects has been slightly affected by their diet. One of the subjects (V. S.) had an elevation of the non-protein nitrogen even before the meat diet was commenced, although urine analysis and other tests for renal function failed

to reveal any abnormalities. This high level was constant throughout the study. The blood uric acid of both men showed a rise of about 2 mg. per 100 cc. in the first 3 months, but returned to the normal at the end of that period even though the same diet was continued for about 5 months after the rise was observed.

The cholesterol values of both subjects were high. K.A. had a maximum of 800 mg. per 100 cc. on one occasion. This increase did not persist after the meat diet was discontinued and is there-

TABLE IV.

*Data Obtained on Subject V. S.*

Blood figures are expressed in mg. per 100 cc., $CO_2$-combining power in volumes per cent.

| Date. | Plasma proteins. | Albumin. | Globulin. | Cholesterol. | A·G ratio. | $CO_2$-combining power. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1928* | | | | | | | |
| Feb. 27 | 6.44 | 4.41 | 2.03 | 263 | 2.17 | 56.0 | Before meat diet. |
| Mar. 2 | 6.54 | 4.41 | 2.13 | 315 | 2.08 | 60.0 | |
| Apr. 20 | 6.25 | | | 307 | | 68.1 | |
| May 28 | 6.69 | 3.62 | 3.07 | 286 | 1.18 | 58.9 | |
| June 25 | 6.81 | 3.64 | 3.17 | | 1.15 | 58.9 | |
| Aug. 25 | 6.70 | 3.52 | 3.18 | 300 | 1.11 | | |
| Oct. 19 | 6.70 | 3.58 | 3.12 | | 1.14 | 60.1 | |
| Dec. 12 | 6.65 | 3.60 | 3.05 | 226 | 1.19 | 53.2 | |
| *1929* | | | | | | | |
| Jan. 23 | | | | 235 | | | |
| Mar. 6 | 6.87 | 3.62 | 3.25 | 212 | 1.18 | 55.1 | |
| Apr. 4 | 6.69 | 3.58 | 3.12 | 218 | 1.14 | 59.0 | After general diet 2 wks. |

fore to be attributed to the large quantity of ingested fat. A visible lipemia was likewise noted.

During the consumption of large quantities of fat a ketonuria was present. The ketone bodies excreted varied from 0.5 to 10 gm. (2) daily, and might be expected to reduce the alkaline reserve; nevertheless the $CO_2$-combining power remained within normal limits. In spite of the constant excretion of ketone bodies in the urine the subjects at no time showed evidence of ketone intoxication either clinically or by laboratory tests.

## SUMMARY.

1. Two healthy men lived exclusively for 1 year on lean and fat meat.

2. The chemical composition of the blood was little affected by such a diet except for a lipemia and hypercholesterolemia. These returned to normal when the diet was discontinued.

3. The uric acid content rose and after about 3 months fell although the diet was continued for 5 months after the elevation had been noted.

4. The $CO_2$-combining power remained within normal limits in spite of the daily ketonuria over the entire period of the experiment.

5. There were no changes in the constituents of the blood that might suggest that renal damage had occurred.

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# THE DETERMINATION OF pH AND CARBON DIOXIDE ON A SINGLE SMALL SAMPLE OF BLOOD PLASMA OR SERUM.

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Economy of blood is always desirable provided the determination gives the required accuracy. Children's veins are difficult to puncture; in infants the longitudinal sinus must be used to obtain a reasonable quantity of blood without stasis. Small quantities, 1 to 2 cc., can be obtained from the heel by Drucker and Cullen's method (1). In small animals, such as the rat, it is necessary to utilize methods that economize material or else use the pooled blood of several animals. At present there are no reports on the acid-base equilibrium of single rats.

Van Slyke's method (2, 3) for $CO_2$ determinations leaves little to be desired for elegance and accuracy; Hastings and Sendroy's method (4), though recently shown to involve variations from the electrometric method which are at present not understood, has probably never led to any false clinical interpretation Eventually these variations will be understood and corrected, for the method is simple and free from errors of manipulation. Moreover, the electrometric method may be subject to large errors when made by the refill technique, and as usually carried out requires several cc. of material.

To combine these two methods on a single sample of material, it is necessary only to measure the pH colorimetrically in 0.9 per cent saline and then, without exposure to air, to transfer the material to the Van Slyke gas apparatus.

## Apparatus.

All manipulation can be made with a single piece of apparatus, shown in Fig. 1. A similar device has been described (5, 6) for

the measurement of the pH of cerebrospinal fluid and blood. It is made from one of the test-tubes used for the color comparison to insure uniformity of size and color of glass. A three-way stop-cock is sealed at the top carrying a capillary tube upwards. This is the measuring pipette, *to contain* 0.1 or 0.2 cc. It is calibrated by mercury above the stop-cock. The delivery tip is made to fit the cup of the Van Slyke apparatus and is used with a rubber guard. The apparatus needs only one mark, 20 times that of the contents of the capillary tube (2 or 4 cc.).
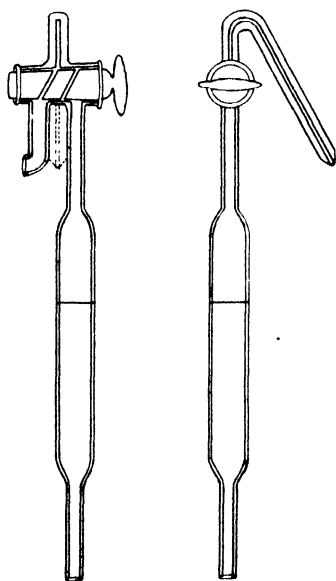


FIG. 1.

*Procedure.*

The saline indicator solution (10.5 cc. of 0.0075 per cent phenol red per 100 cc.) is first evacuated in the Van Slyke machine. Such saline needs no addition of alkali. It is more free of $CO_2$ than that used by Stadie (7) or Cullen (8). The apparatus, including the stop-cock, is filled with mercury. The plasma or serum, obtained by centrifugalization under oil in stoppered tubes, is transferred to the pipette by suction. The stop-cock is turned to

communicate to the side arm (as is shown in the illustration). The sample is drawn in, and then sealed with a half turn of the stop-cock. The delivery end is placed under the surface of the extracted saline. The stop-cock is made to communicate with the bulb of the apparatus, and the $CO_2$-free saline is drawn to the mark by lowering the levelling bulb. The apparatus is then placed in a suitable bath at 38°. The solution is compared with Hastings and Sendroy's bufferless pH standards, and the pH thus determined.

TABLE I.

*Factor for Calculating $CO_2$ in Volumes Per Cent.*

Sample = 0.2 cc., a* = 0.5 cc., i = 1.037.

| S† | 23° | 24° | 25° | 26° | 27° | 28° |
|---|---|---|---|---|---|---|
| cc. | | | | | | |
| 2.0 | 0.325 | 0.324 | 0 323 | 0.321 | 0.319 | 0.318 |
| 2.5 | 0.328 | 0.326 | 0.325 | 0.324 | 0.322 | 0.321 |
| 3.0 | 0.331 | 0.329 | 0.328 | 0.327 | 0.325 | 0.323 |
| 3 5 | 0 334 | 0.332 | 0.331 | 0.330 | 0.328 | 0.326 |
| 4 0 | 0.337 | 0.335 | 0.334 | 0.332 | 0.330 | 0.329 |
| 4 5 | 0 341 | 0.339 | 0.337 | 0.335 | 0.333 | 0.332 |
| 5 0 | 0 344 | 0.342 | 0.340 | 0.338 | 0.336 | 0.335 |

These symbols have the same significance as those used by Van Slyke and collaborators.

* a indicates the volume at which the gas is measured; i is the absorption factor.

† S is the total volume of fluid.

The determination of $CO_2$ then follows. The capillary of the apparatus may be filled with either saline or mercury. A rubber guard is placed on the delivery tip. The Van Slyke pipette has been previously filled with mercury. In addition 2 to 3 cc. are placed in the cup; its levelling bulb is in the lower position and remains there throughout the delivery; its three-way stop-cock is closed. A rubber guard on the delivery tip of the Shohl pipette is then pressed firmly into the base of the cup and is thus covered with mercury, while the levelling bulb is held in the palm of the hand. The three-way stop-cock of the Van Slyke pipette is opened. If the seal is tight no mercury flows. The three-way stop-cock of the Shohl pipette is then turned to deliver. The rate of flow is controlled, preferably by this stop-cock. The sample followed

by mercury is thus delivered into the Van Slyke pipette. The stop-cock is closed while pressure is still maintained upon the rubber guard. The stop-cock of the Van Slyke pipette is closed and the transfer is complete. 0.1 cc. of n lactic acid and a drop of octyl alcohol are then introduced. A mercury seal is made. The determination is then made according to the Van Slyke technique (3).

The calculation of the amount of gas from the pressure readings is then made according to the revised equations of Van Slyke and Sendroy (9). For convenience the factors have been calculated for varying temperatures and volumes of diluent. These are given in Table I.

<center><em>Results.</em></center>

The method was first checked on carbonate solutions according to the technique recommended by Van Slyke and Sendroy (9). The results checked the theoretical values within 0.4 per cent for 1 cc. and 2 per cent for 0.15 cc. samples. This accuracy is not as good as obtained by the above authors with 2 cc. samples in the 100 cc. machine. They do not give data regarding the accuracy to be obtained with 0.2 cc. The method of delivery by mercury has, however, been tested by them and found accurate for delivery of amounts of fluid used above within the experimental error. We believe that the accuracy of a pipette *to deliver* between marks for volumes of 0.2 cc. or less is inferior to the method here outlined. Delivery of a pipette calibrated with water gave the value of 1.3 per cent too low for serum.

The use of saline instead of water affects the solubility of CO₂ in the solution. Calculations from the data of Van Slyke and collaborators (10) and actual determinations show that this falls within the experimental error. Under our conditions the effect is of the order of 1 part in a thousand.

The last 50 determinations on serum obtained from the longitudinal sinus have been determined by using 1 cc. of serum according to Van Slyke and Sendroy's technique or by the author's technique. The agreement between the two has been 2 per cent or slightly better. pH determinations by this method and by Hasting's method showed no differences.

SUMMARY.

When economy of blood is necessary, the method described above allows a determination of both the $CO_2$ and pH on 0.1 to 0.2 cc. of blood serum or plasma.

I am indebted to my son Theodore Shohl for the attached drawing.

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# A MICRO ELECTRODE AND VESSEL FOR THE DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF BLOOD MEDIA, WHOLE BLOOD, AND OTHER BIOLOGICAL FLUIDS.

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### INTRODUCTION.

It is well known that the determination of the hydrogen ion concentration of whole blood by the electrometric method is an exceedingly difficult matter. This is due, especially, to the presence of carbonate and oxyhemoglobin.

If hydrogen is allowed to bubble through blood or if the overlying atmosphere is replaced by this gas, carbon dioxide will be quickly removed, thus lowering the hydrogen ion concentration. On the other hand, if hydrogen is not continuously passed through or over the blood, the $CO_2$ will not only reduce the partial pressure of hydrogen surrounding the electrode but the oxygen in combination with the hemoglobin will be given off and cause a depolarization of the electrode. When oxyhemoglobin loses oxygen the pH is increased, as oxidized hemoglobin behaves as a weak acid. Whether $CO_2$ or oxygen or both are given off, the pH is increased; that is, the blood becomes more alkaline in reaction. In order that the true reaction may be obtained precautions must be taken to see that the pH readings are made before there is any appreciable loss of these two gases.

Michaelis and Rona (1) showed many years ago that equilibrium is quickly reached if the minimum of contact is obtained between the solution and electrode, previously saturated with hydrogen gas.

It is the purpose of this article to describe a micro electrode and vessel which produce a minimum of contact between the solution and electrode in a very easy and rapid manner. No shaking is required. Furthermore, hydrogen is continuously passing through

the vessel without in any way bubbling through the solution. Only a few drops of blood or other fluid are required for each determination. Readings are made almost instantaneously before there is any appreciable loss of carbon dioxide and oxygen. The apparatus is simple in operation. It is believed that this method possesses superiority over any now in use for the electrometric determination of the hydrogen ion concentration of blood or other biological fluids. Practically perfect agreements are obtained when checked by the colorimetric method.

*Description of Apparatus.*

The apparatus is shown in Fig. 1.

*1. Hydrogen Electrode.*—The hydrogen electrode (A) (Fig. 1) consists of a 32 gage platinum wire sealed into a piece of tubing. The tubing has an outer diameter of 4 mm. and a 2 mm. bore. Contact is made by means of mercury. The tube may be conveniently filled with mercury by means of a hypodermic syringe and needle. The length of the platinum wire is 6 mm.

The electrode is surrounded by another tube (B) similar to a Hildebrand vessel but much smaller in dimensions. The tube has an outside diameter of 6.5 mm. and a bore of 5 mm. A tube 25 mm. long and 10 mm. outside diameter is sealed onto the lower end. The total length of (B) is 72 mm. This tube is slipped through a 1-hole No. 2 rubber stopper and a side tube (C) of the same diameter sealed on. Tube (B) is placed about 4 mm. above the opening of the feeder (F).

The hydrogen electrode (A) has sealed on its upper end, by means of De Khotinsky wax, a brass head-piece (E) threaded at its lower end. This head-piece screws into another brass piece (D) threaded on the inside and sealed on the upper end of tube (B). The purpose of this is to regulate the distance of the platinum wire from the opening of the feeder (F).

*2. Electrode Vessel.*—The rubber stopper sits inside of the upper end of the larger vessel (G), 65 mm. long and 16 mm. in internal diameter. A small hole (H) is blown into the upper end just below the rubber stopper to allow for the escape of hydrogen. Tube (K) having an outer diameter of 6 mm. and extending 35 mm. below the opening (S) is sealed in, 10 mm. above the lower end.

*3. Capillary Feeder.*—A capillary tube (L) is held in place at the
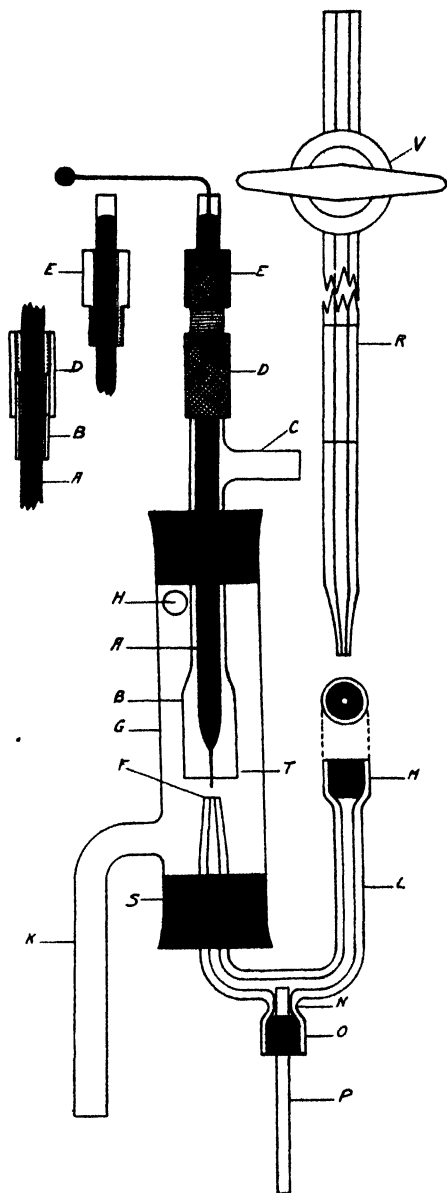
FIG. 1. Micro electrode and vessel, as described in text.

lower end of the vessel (*G*) by means of a 1-hole No. 2 rubber stopper.   The capillary is 5 mm. in external and 2 mm. in internal diameter.   The tube is tapered at the end (*F*), giving an external diameter of 3 mm. and 1 mm. bore.   A larger sized tube (*M*) having an external diameter of 8 mm. is sealed on the upper end of tube (*L*).   This holds a small piece of rubber tubing having a bore of 2 mm.   Tube (*N*) is also sealed onto (*L*) and has an internal di-
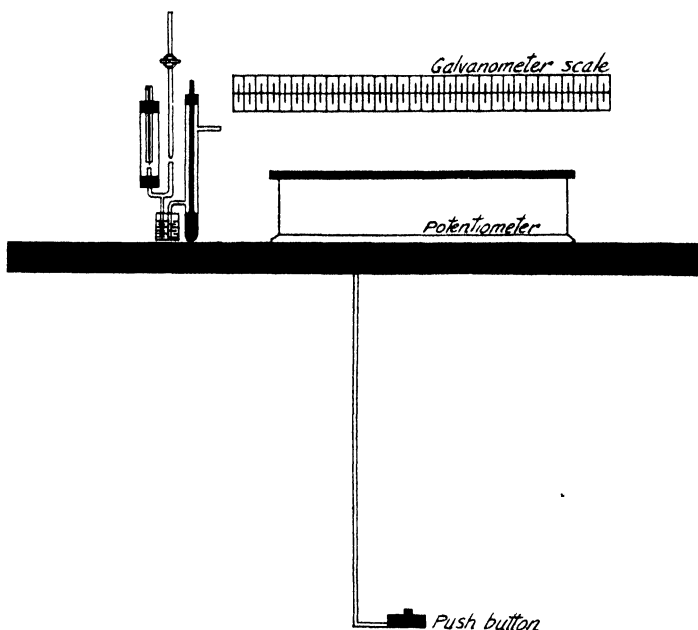


FIG. 2. Arrangement of the potentiometric circuit.

ameter of 3.5 mm.   A tube (*O*), similar to (*M*) in all respects, is sealed onto (*N*).   An agar bridge (*P*) is inserted into (*O*) and (*N*), making connection with the solution flowing through (*L*).   This bridge has an outer diameter of 3 mm.

*4. Micro Burette.*—This consists of a 1 cc. pipette (*R*) having a glass stop-cock (*V*) sealed onto the upper end.   The tip is drawn out until the bore has a diameter of about 0.4 mm.

*5. Potentiometer.*—The potentiometer circuit (shown in Fig. 2) is closed by means of a foot arrangement.   Wires are soldered to

the two poles of the key within the potentiometer and then to the two binding posts of a door bell push button fastened to the floor. The potentiometer is a Leeds and Northrup ion potentiometer having an accuracy of 0.005 to 0.01 pH unit.

*6. Galvanometer.*—A Leeds and Northrup type R reflecting galvanometer with lamp and scale is employed. This type proved to be very convenient to read and possessed the desired sensitivity for this kind of work.

*7. Platinizing the Electrodes.*—For satisfactory results a light coating of platinum black must be used. If the deposit is too light or too heavy, the readings will be low. Clark (2) states that the inclination is to make the deposit too thick with the result that a sluggish electrode is produced. The best practice is to allow the electrode to remain in the platinic chloride solution until the glint of polished metal can no longer be seen. This requires only a few seconds. Very sensitive electrodes are produced in this manner, which reach equilibrium practically immediately.

In dealing with biological liquids and bacteriological media the electrodes should be platinized often. Clark (2) states that, "the deposit of films of protein have been detected as definite causes of electrode (poisoning)."

*8. Purification of Hydrogen.*—Tank hydrogen is used and purified by the method of Cullen (3), which consists in passing the gas through solutions of mercuric chloride, alkaline potassium permanganate, alkaline pyrogallol (twice), dilute sulfuric acid, and finally distilled water. This method of purification is convenient and the washed gas is very satisfactory.

### Operation of Micro Electrode and Vessel.

The short agar bridge (*P*) is inserted into (*O*) and (*N*). The opening of tube (*K*) is closed by means of a small stopper and distilled water is run into the vessel (*G*) at (*M*) to the mark (*T*), by means of a narrow-tipped 10 cc. pipette. Hydrogen is introduced at (*C*) and allowed to bubble through the distilled water for a few minutes or until the electrode is saturated. It is very important that hydrogen bubble through the water at the rate of about six bubbles per second. If a vigorous stream of hydrogen is not used the electrode will not come to equilibrium immediately when in contact with the solution to be examined.

The solution to be examined is drawn into the pipette $(R)$. The tip of the pipette is inserted into the rubber tube contained in $(M)$, and then clamped to a ring-stand. The potentiometer is standardized with respect to the standard cell and the dials set at about the position where it is believed the pH will fall.

The stopper in the opening of tube $(K)$ is removed to permit the water in the vessel $(G)$ to run out. Stop-cock $(V)$ is turned until the water in tube $(L)$ is replaced by the solution to be examined. The metal head-piece $(E)$ is screwed either to the right or left until the platinum electrode just makes contact with the solution. The best plan is to screw the electrode down until it dips into the solution and then screw up as far as possible. It is absolutely essential that a minimum of contact is obtained between the electrode and solution; otherwise the readings will be low.

To make a determination, the pipette is manipulated with the left hand, the potentiometer dial with the right hand, and the potentiometer key with the foot. The stop-cock of the pipette is opened and a small drop of liquid allowed to run out. This is carried out by opening and quickly closing the stop-cock. Simultaneously contact is made with the foot and the potentiometer dial adjusted. Another small drop of solution is introduced and the manipulations repeated. The potentiometer is quickly adjusted to catch the point of lowest reading. Usually three or four determinations are required to find the lowest reading.

After a satisfactory result is obtained the liquid is washed out and the electrode again saturated by filling $(G)$ with distilled water to the mark $(T)$. The electrode is now ready for another sample.

It can be seen that this electrode and vessel possess marked advantages over other types where a minimum of contact is necessary. If the electrode dips too far into the solution, low readings are obtained. On the other hand, if the electrode does not touch the solution, more must be run in. During this delay $CO_2$ and $O_2$ are rapidly given off, before a reading is made, with the result that the pH is too high. With this electrode it is not possible to run too much liquid into the feeder as the excess spills over the sides and runs out. The size of the drop remains the same. Contact is automatically made between the solution and electrode. Once the electrode is adjusted for a particular solution, it need not be disturbed.

At no time does the hydrogen bubble through the solution. Furthermore the gas is continuously passing into the vessel to keep the electrode saturated. The hydrogen escapes at $(H)$.

By means of the foot arrangement the potentiometer contact is made practically simultaneous with the appearance of the drop of blood or other fluid on the opening $(F)$. The galvanometer swing is recorded almost immediately. The potentiometer dial is quickly set and the operation repeated with another small drop of

TABLE I.

*Comparison of pH Values by the Electrometric and Colorimetric Methods.*

| Material | pH by electrometric method | pH by colorimetric method. |
|---|---|---|
| Buffer solutions. | 3.96 | 3.97 |
| | 6.41 | 6.41 |
| | 7.55 | 7.55 |
| | 6.60 | 6.60 |
| Rabbit blood. | 7.59 | 7.60 |
| | 7.55 | 7.56 |
| | 7.52 | 7.52 |
| | 7.45 | 7.46 |
| | 7.47 | 7.45 |
| | 7.49 | 7.47 |
| | 7.42 | 7.45 |
| Hemolyzed blood media. | 7 32 | 7.30 |
| | 7.30 | 7.28 |
| Raw milk. | 6.43 | 6.43 |
| Autoclaved milk. | 6.05 | 6.04 |
| | 6.08 | 6.08 |

liquid. The lowest reading is usually obtained after three or four determinations. After this it makes no difference how many readings are made, the results will be the same.

This method removes any of the objections due to the potentiometer key being pressed with the hand. No time is lost during the operation. The readings are practically instantaneous. The foot contact is made just as the stop-cock is turned with the left hand to introduce a fresh drop of liquid at $(F)$. The potentiom-

eter is easily and quickly manipulated with the right hand. A sensitive galvanometer with a lamp and scale reading device adds greatly to the accuracy of the determinations.

After several determinations grease may collect on the opening of the feeder (*F*). This causes the drops to become too large. This may be remedied by removing the upper rubber stopper and rubbing the surface of the opening with a cotton swab dipped in either ether or chloroform.

It is needless to state that the electrode is checked against a standard solution before and after each determination. For this purpose 0.05 N potassium acid phthalate is used.

In Table I are shown results obtained by the electrometric and colorimetric methods, with various fluids. The procedure, as recommended by Levy, Rowntree, and Marriott (4) is used. It is seen that the agreements are as good as can be expected when dealing with biological liquids.

### CONCLUSIONS.

1. A micro electrode and vessel are described for the determination of the hydrogen ion concentration of blood and other biological fluids.

2. The vessel is so constructed that a minimum of contact is automatically obtained between the fluid and electrode.

3. With the aid of a foot arrangement readings are practically instantaneous, before there is any appreciable loss in the concentration of carbon dioxide and oxygen.

4. The results obtained show very good agreement with the colorimetric method.

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# NOTE ON TRIMETHYL-α-GLUTAROBETAINE.

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In connection with other investigations it was found desirable to know something of the properties of trimethyl-α-glutarobetaine. While a number of workers have investigated the action of alkylating agents on glutamic acid, definite products with the exception of certain gold or platinum salts of doubtful composition seem not to have been isolated. Engeland (1) through the action of methyl iodide and potassium hydroxide on glutamic acid obtained two gold salts which were regarded as possibly derivatives of *N*-dimethylglutamic acid and its dimethyl ester. Novák (2) using dimethyl sulfate as alkylating agent failed to detect Engeland's gold salts but obtained platinum and gold salts which were supposed to be derived from the dimethyl ester of *N*-trimethylglutaminic acid. Ackermann and Kutscher (3) also using dimethyl sulfate obtained a gold salt of still different properties which they regarded as derived from trimethyl-α-glutarobetaine I.

$$
\begin{array}{ccc}
\text{COOH} & \text{CO}\!-\!\text{O} & \text{COOH} \\
| & | \quad \quad & | \\
\text{CH}_2 & \text{CH}_2 \quad & \text{CH}_2 \\
| & | \quad \quad & | \\
\text{CH}_2 & \text{CH}_2 \quad & \text{CH}_2 \\
| & | \quad \quad & | \\
\text{CH} \cdot \text{N(CH}_3)_3 & \text{CH}\!-\!\text{N(CH}_3)_3 & \text{CHN} \cdot \text{(CH}_3)_3\text{OH} \\
| \quad | & | \quad \quad & | \\
\text{CO}\!-\!\text{O} & \text{COOH} \quad & \text{COOH} \\
\text{I.} & \text{II.} & \text{III.}
\end{array}
$$
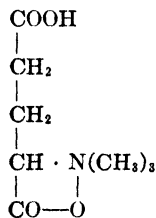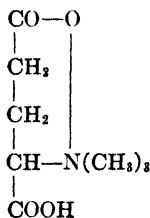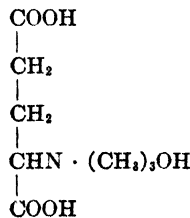
No evidence was adduced as to the structure of the acid, which they did not isolate and it may be noticed that it might equally well be a γ-betaine as shown in Formula II. As a matter of fact

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the product to be described retains a molecule of water more than that required for Formulas I and II, even after drying *in vacuo* over phosphorus pentoxide at 50°. It may therefore be assumed that the free acid has the open chain formula of an ammonium derivative as shown in Formula III.

Glutamic acid (20 gm.) was suspended in 50 cc. of water and dimethyl sulfate (100 gm.) and 33 per cent sodium hydroxide (100 cc.) added alternately. The solution was maintained slightly on the alkaline side and was cooled occasionally if the temperature rose above 50°. After the dimethyl sulfate was all decomposed, the solution was diluted to 800 cc., acidified with 30 cc. of strong sulfuric acid, and then precipitated with phosphotungstic acid. The latter portions of the precipitate are very finely crystalline. The phosphotungstate was filtered off, dissolved in aqueous acetone, decomposed by baryta in the usual way, and the filtrate exactly freed from barium with sulfuric acid. The strongly acid reacting solution was then concentrated to about 40 cc. and allowed to crystallize in a cool place. The crystals which form large white glistening prisms are rubbed up with a little alcohol and filtered off. The syrupy mother liquor gives additional crops on careful concentration. In a good experiment the yield of crystalline acid amounts to 12 to 13 gm., while a considerable amount remains in the mother liquor. The substance which melts at 211–213° is extremely easily soluble in water but insoluble in alcohol and ether. For analysis it was dried *in vacuo* over phosphorus pentoxide.

0 1298 gm substance: 0.2248 gm $CO_2$ and 0.0945 gm. $H_2O$.
0 200 " " : 0 01344 gm N (Kjeldahl).
  $C_8H_{17}O_5N$. Calculated. C 46.4, H 8.21, N 6 76.
            Found.    " 47 2, " 8 08, " 6.72.

Trimethyl-α-glutarobetaine is dextrorotatory in aqueous solution. Three different preparations gave the following results.

| c | l | α | $[\alpha]_D^{20°}$ |
|---|---|---|---|
| 2 0 | 2 2 | +0 52 | +11 8 |
| 17 0 | 2 2 | +4 50 | +12 0 |
| 34 7 | 1 0 | +4 25 | +12 2 |

The acid titrates sharply as a monobasic acid with phenolphthalein, 0.2 gm. requiring 9.90 cc. of decinormal sodium hydroxide as against a calculated amount of 9.7 cc.

Crystalline salts have been obtained with alkaloids and with platinum and gold chlorides. The salts with strychnine, brucine, cinchonine, and quinine are all finely crystalline but so soluble in water and most solvents as to be of little value for purposes of identification. Picric acid does not yield a crystalline picrate under ordinary conditions. The silver, barium, and mercury salts are all extremely soluble. Mercuric acetate and soda give no precipitate nor does alcoholic zinc chloride. The phosphotungstate is sparingly soluble and crystallizes from dilute sulfuric acid in long colorless needles. The phosphomolybdate separates in the form of thin golden plates. Potassium triiodide in the presence of dilute sulfuric acid gives a blackish brown oil slowly changing to long needles. The hydrochloride, perchlorate, and chromate are all very soluble in water.

The gold salt is very readily soluble in hot water and moderately soluble in cold water. It separates in the form of long thin needles which contain water of crystallization. After drying at 70–80°, the recrystallized salt melts at 139–140°. It is probably identical with that described by Ackermann and Kutscher as melting at 135° for the less pure specimens melted between 135–139°.

*Analysis* 0 1228 gm gave 0 0458 gm Au
$C_8H_{15}O_4N$ $HAuCl_3$     Calculated     37 3 Au     Found     37 3 Au

The platinum salt is extremely soluble in water and can only be conveniently prepared in strong alcoholic solution. It was recrystallized from absolute alcohol and separated in radiating clusters of needles melting, after drying at 70°, at 204–205°.

*Analysis.* 0.1271 gm gave 0 0311 gm platinum
$(C_8H_{15}O_4N)_2H_2PtCl_6$     Calculated 24 7 Pt     Found     24 5 Pt

Trimethyl-$\alpha$-glutarobetaine gives only a faint pyrrole reaction on dry distillation with zinc dust. On warming with strong caustic potash, little or no trimethylamine is evolved and glutaconic acid is not formed. The substance is clearly remarkably stable.

It would be of interest to ascertain whether "schlempe" molasses

from beet sugar manufacture, which is so rich in ordinary betaine and glutamic acid, contains trimethyl-α-glutarobetaine.

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# ARACHIDONIC ACID IN THE LIPIDS OF THYROID, SUPRARENAL, AND SPLEEN.*

By J. B. BROWN.

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In continuation of the work reported in a previous communication (1) in which it was shown that arachidonic acid, $C_{20}H_{32}O_2$, is the only highly unsaturated fatty acid in liver lipids, a similar study has been made of the lipids of thyroid, suprarenal, and spleen. No chemical investigations of the nature of the fatty acids in these tissues have been been previously reported. Lipid preparations from these glands have been hydrolyzed, the fatty acids recovered and converted into methyl esters, and these esters distilled. The results of analysis of the fractions and isolation and analysis of the ether-insoluble bromides which give a bromine content and melting point agreeing satisfactorily with the theory for methyl octobromoarachidate show that arachidonic acid is likewise the only unsaturated acid with three or more double bonds present in these tissues. Bloor (2) was unable to find linolenic acid in the lipid fractions of beef brain, liver, kidney, lung, and pancreas, and Theis (3) obtained similar results on normal and abnormal liver tissues.

## Estimation of Arachidonic Acid.

In discussion of the results on liver lipids (1), mention was made of two available methods for the calculation of the amount of arachidonic acid present. Both of these were based on the weight of ether-insoluble bromide obtained when a given sample is brominated and thoroughly washed in cold ether. In the first method the calculation was made by multiplying the polybromide

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number by the acid content of the bromide.  The calculation
would be represented by the equation,

$$\text{Per cent arachidonic acid} = \text{polybromide No} \times \left(\frac{100 - \text{per cent Br}}{100}\right)$$

This method so far as it goes is undoubtedly correct.   The result
certainly represents a quantity of arachidonic acid which is present,
but, in the writer's opinion, is far too low.   In order that the result
may be accurate a theoretical yield of bromide must be obtained.
This brings up the question of the isomeric bromides of the
fatty acids.   When methyl arachidonate is brominated it yields
methyl octobromoarachidate.   Each carbon atom to which a
bromine atom is attached is asymmetric, so that there is a possibil-
ity of 256 isomers.   Those isolated in the determination of poly-
bromide number are the compounds insoluble in cold ether.   Ex-
periment shows that in brominating 1 gm. of pure methyl arachi-
donate only 0.776 gm. of bromide can be isolated (1), whereas ac-
cording to the theory there should be 3.01 gm.   Apparently,
therefore, about one-fourth of the above isomers is insoluble in
cold ether, the remainder being soluble and probably liquid bro-
mides.   Similar results are obtained when linolenic acid and its
methyl ester are brominated, the latter giving a polybromide
number of only 58.1 (4).   The pure methyl esters of the mixed
highly unsaturated fatty acids of menhaden oil give a poly-
bromide number of 105, of cod oil 125, and of herring oil 113 (5),
which results are approximately one-third of the theory.

The second method (1) was employed to take into account both
isomerism and solubility and was based on the fact that pure
methyl arachidonate gives a polybromide number of 77.6.

$$\left.\begin{array}{c}\text{Per cent arachi-} \\ \text{donic acid}\end{array}\right\} = \frac{\text{polybromide No.} \times 100}{77.6} = \text{polybromide No.} \times 1.29$$

This simply means that a sample of methyl ester giving a poly-
bromide number of 10 would contain 12.9 per cent of arachidonic
acid.   The method has the disadvantage of being dependent on
the assumption that the arachidonic acid present in the original
lipid or its resultant methyl ester is the same chemical individual
or the same mixture of isomeric acids as is present in the pure
product prepared by reduction of the bromine addition products

with zinc. The result given by the method, therefore, is only an approximation but, in the writer's opinion, is more nearly accurate than that given by the first method. Calculations of arachidonic acid in the experimental work which follows are based on the latter method.

*Lipids.*—The lipids from pig thyroid and beef suprarenal and spleen were kindly furnished through the courtesy of Dr. David Klein of the Wilson Laboratories. They were prepared from glands, which had been desiccated in a vacuum oven at low temperature, by extraction with benzene and subsequent removal of the solvent. In general they were similar in appearance, chocolate-brown in color and semisolid at ordinary temperature.

*Saponification.*—The lipids were saponified in 600 gm. lots as described previously (1) by treatment for a short time with a mixture of a solution of 400 gm. of KOH in 400 cc. of water and 500 cc. of alcohol. Saponification was apparently complete in 5 minutes, but in most instances was continued for 30 minutes under the reflux. After dissolving the soaps in 2 liters of hot water, they were decomposed with an excess of HCl. The contents of the flask were boiled until the fatty acids separated to a clear layer.

*Preparation of Methyl Esters.*—The fatty acids of the thyroid were warmed under reduced pressure until all water was removed, and then refluxed with twice their weight of methyl alcohol containing dry HCl in solution. In the cases of suprarenal and spleen fatty acids, however, water was held apparently in solution in the fatty acid layer. Attempts to remove it by vacuum distillation failed on account of foaming. This difficulty was overcome by the addition of toluene and small quantities of *n*-butyl alcohol. Upon distillation under reduced pressure, the water came over with the toluene. More toluene was added until the distillate came over clear. The dehydrated fatty acids were then esterified as usual. After refluxing for at least 18 hours with methyl alcohol, the esters were cooled, and poured into 2 volumes of cold water. Addition of a small amount of salt or of butyl alcohol facilitated separation into two layers. As before, however, the upper ester layer could not be easily dehydrated, and it was necessary to add

toluene and distil to remove the last traces of water. The esters were rapidly distilled under reduced pressure. The yield of esters

TABLE I.

*Analysis of Methyl Esters of Glandular Lipids.*

| Source. | Yield of esters. | Mean molecular weight of acids. | Iodine No. | Poly-bro-mide No. | Br | Arachidonic acid. | Melting point of bromides |
|---|---|---|---|---|---|---|---|
| | *per cent* | | | | *per cent* | *per cent* | °C. |
| Liver . . ......... . | 83 | 274.9 | 71.85 | 6.0 | 66.48 | 7.7 | |
| Thyroid ......... . .. | 94 | 267.4 | 60.70 | 0.3 | 65.26 | 0.4 | 230–233 |
| Suprarenal ........... | 72 | 272.6 | 60.27 | 4.3 | 66.10 | 5.5 | 228–229 |
| Spleen . .. ..... ...... | 54 | 273.1 | 57.01 | 3.1 | 66.15 | 4.0 | |

TABLE II.

*Results of Fractionation of Methyl Esters of Glandular Lipids.*

| Fraction boiling point. | Weight. | Mean ecular ght of ids. | Iodine No. | Polybro-mide No | Br | Melting point of bromides. |
|---|---|---|---|---|---|---|
| | | | | | | |

Pig thyroid, 7 to 8 mm. pressure.

| °C. | *gm.* | | | | *per cent* | °C. |
|---|---|---|---|---|---|---|
| 170–180 | 195 | 259.1 | 49.21 | 0.04 | | |
| 180–190 | 154 | 267.7 | 61.59 | 0.17 | | |
| 190–200 | 129 | 273.0 | 72.42 | 0.31 | | |
| 200–210 | 15 | 285.3 | 83.38 | 2.80 | | |

Beef suprarenal, 10 mm. pressure.

| 188–196 | 91 | 264.8 | 40.52 | 1.31 | 66.34 | 230 |
|---|---|---|---|---|---|---|
| 196–200 | 144 | 272.5 | 51.90 | 2.63 | 65.31 | 227–228 |
| 200–206 | 117 | 280.1 | 70.10 | 3.92 | 66.05 | 228 |
| 206–235 | 36 | 294.5 | 110.0 | 17.41 | 66.70 | 228–230 |

Beef spleen, 10 mm. pressure.

| 182–191 | 75 | 261.9 | 38.00 | 1.13 | 64.42 | 228–232 |
|---|---|---|---|---|---|---|
| 191–196 | 95 | 270.0 | 48.55 | 1.61 | 66.50 | 229–230 |
| 196–200 | 74 | 276.6 | 63.63 | 2.48 | 66.73 | 229–230 |
| 200–220 | 34 | 288.0 | 89.20 | 12.00 | 66.96 | 228–232 |

based on the weight of original lipids used was: thyroid 94 per cent, suprarenal 72 per cent, spleen 54 per cent. It is apparent, there-

fore, that the lipids of thyroid are largely of the nature of ordinary fats, while those of suprarenal and spleen are more complex in nature, containing large amounts of lecithins and similar compounds. Haeberli (6) on the basis of staining reactions concluded that the lipids of thyroid were chiefly neutral fat. In all three cases the esters were redistilled and analyzed. The results are given in Table I. For comparison the previous results from liver lipids are included.

*Distillation of Methyl Esters.*—The methyl esters were distilled from a 1 liter Claissen flask upon the side-arm of which was attached a short Pyrex condenser jacket and which delivered into a specially devised Pauly receiver which was constructed from a standard Pyrex suction flask (7). About 1 hour was required for each distillation. The quantities of esters distilled were as follows: thyroid 500 gm., suprarenal 400 gm., spleen 282 gm. Four fractions were cut in each case. The results in Table II give the weights of the fractions and the analytical data for each.

### DISCUSSION.

While it was not the intention of this work actually to estimate the kind and amount of fatty acids present other than arachidonic, nevertheless, upon examining the data one is immediately struck by the similarity of the fatty acids from the three sources. The mean molecular weights of the mixed acids differ by only 5.7 units and the iodine numbers of the mixed esters by no more than 3.7 units. The molecular weights of the acids in the lowest boiling fractions vary from 259–264 which is only three to eight points too high for palmitic acid (256). In every case the highest boiling fraction gives a molecular weight higher than the theory for stearic acid (284), demonstrating the presence of acids higher than the $C_{18}$ series. On the basis of these results it is quite probable that the fatty acids consist mainly of a mixture of palmitic, stearic, oleic, and arachidonic acids. Acids of series lower than $C_{16}$ may also be present.

Ether-insoluble bromides were obtained not only from the original mixed esters in each case but also from each fraction of these esters. None of these bromides gave evidence for the presence of methyl hexabromostearate which melts at 155°. On the other hand in every instance but one the bromine content

of the bromides was lower than the theory for methyl octobromoarachidate, 66.78 per cent. The exception noted (fourth fraction for spleen) gave a result only 0.18 per cent too high, which may have been due to experimental error or to admixture with octobromoarachidic acid which contains 67.78 per cent bromine. Therefore no acids more highly unsaturated than arachidonic are present in appreciable quantity. The melting points of the bromides likewise agree satisfactorily with that for methyl octobromoarachidate, i.e. 228–230°. The fact that the bromine content of the bromides was in several instances a little low is due to the fact that they were prepared by washing four times with ether and separation each time by centrifugation. Under these conditions one would expect the results to be low. From these data it is concluded that arachidonic acid occurs as the sole highly unsaturated fatty acid in the lipids of thyroid, suprarenal, and spleen and in the following amounts respectively, thyroid 0.4 per cent, suprarenal 5.5 per cent, and spleen, 4.0 per cent of the total fatty acids.

### SUMMARY.

1. The methods of estimating arachidonic acid from the weight of ether-insoluble bromide are discussed.

2. The lipids of thyroid are mostly neutral fats; those from suprarenal and spleen contain large amounts of more complex substances.

3. The fatty acids of thyroid, suprarenal, and spleen lipids consist largely of palmitic, stearic, and oleic acids.

4. Arachidonic acid is the only highly unsaturated fatty acid occurring in thyroid, suprarenal, and spleen; it occurs in the following amounts respectively, 0.4, 5.5, and 4.0 per cent of the total fatty acids.

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# THE OCCURRENCE OF A NEW HIGHLY UNSATURATED FATTY ACID IN THE LIPIDS OF THE BRAIN.*

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The presence of a highly unsaturated fatty acid in the lipids of the brain was reported for the first time by Grey in 1913 (1). Grey hydrolyzed brain tissue with 20 per cent potassium hydroxide, liberated the fatty acids with mineral acids, and separated them into liquid and solid acids by the well known lead soap–ether method. From the analysis of these two fractions the following fatty acids were found: stearic, an isomer of stearic acid melting at 51°, palmitic, myristic, oleic, an isomer of oleic acid melting at 41°, linolic, linolenic, and finally clupanodenic acid, $C_{18}H_{24}O_2$. The presence of this last acid was inferred from the isolation of a bromine addition product, believed to be dodecabromostearic acid, $C_{18}H_{24}O_2Br_{12}$, containing 77.7 per cent bromine. 20 per cent of the solid acids were said to be hydroxy acids, which accounted for the unusually high molecular weight. The evidence which Grey offered for a number of these acids cannot be regarded, however, as conclusive. No one since that time has shown the existence in any tissue of an acid so unsaturated as clupanodenic. This acid should not be confused with clupanodonic, $C_{18}H_{28}O_2$, which occurs in fish oils (2, 3). According to the theory proposed by McGregor and Beal (4), the probability of a naturally occurring $C_{18}$ fatty acid with 6 double bonds is very slight.

From the fatty acids of brain cephalin MacArthur and Burton (5) isolated bromides containing 67.7 to 69.4 per cent bromine. These results were stated to agree better with octobromostearic acid than with octobromoarachidic acid, suggesting the presence

of clupanodonic acid in cephalin.   The authors, however, did not consider the presence of this acid to be proved.

Levene and Rolf (6) identified arachidonic acid, $C_{20}H_{32}O_2$, in brain cephalin and lecithin.   The bromine content of their bromides agreed satisfactorily with the theory for octobromoarachidic acid.

The presence of arachidonic acid in brain tissue was confirmed by Wesson (7) who, however, obtained a number of preparations of bromides of fatty acids which gave results too high for octobromoarachidic acid.   Bloor (8) in an investigation of the lipids of the vital organs of beef found that the content of 4-double bond acids was greatest in the brain.   The other tissues examined were liver, kidney, lung, and pancreas.

In a continuance of studies in this laboratory of the nature of the highly unsaturated fatty acids occurring in glandular lipids, in which it was shown that in the liver (9) and spleen, thyroid and suprarenal (10) arachidonic acid is the only acid of this character occurring in appreciable quantities, the total lipids of beef brains were prepared, saponified, converted into methyl esters, and these esters distilled.   The average molecular weight was found to be considerably higher than with any of the other lipids studied. Distillation in a vacuum gave fractions boiling 50° higher than the others.   Some of the bromine addition products contained from 1 to 1.5 per cent more bromine than the theory for methyl octobromoarachidate.   Furthermore these bromides did not melt below 250°, whereas the latter melts with gas formation at 228–30°.   The ether-insoluble bromides of the total fatty acids of the brain were reduced with zinc and the product converted into methyl esters.   These pure highly unsaturated methyl esters boiled over a range of 58° (200–258°) and by the usual methods of analysis indicated the presence of a mixture of methyl arachidonate with one or more other acids of higher molecular weight and more highly unsaturated than arachidonic acid.   The presence of tetracosapentenoic acid ($C_{24}H_{38}O_2$) is suggested.

### EXPERIMENTAL.

*Brain Lipids.*—Two lots of lipids from beef brains were kindly furnished by Dr. David Klein of the Wilson Laboratories.   They were prepared by extraction of desiccated brain with benzene

and removal of the solvent. Their physical properties differed greatly from those of the other lipids studied in this series; *i.e.*, liver, spleen, suprarenal, and thyroid. In color they were light brown; in feel, a soft wax. They did not melt at 100°, were not completely soluble in ether, but were readily soluble in boiling 95 per cent alcohol.

Two other preparations were made in this laboratory. 10 kilos of fresh beef brains were passed through a meat chopper and completely mixed with 14 liters of cold ·alcohol. After standing overnight with occasional stirring the material was strained through a lard press. The filtrate was found to contain almost no lipid material and was discarded. The press cake was heated to boiling with 10 liters of fresh alcohol and pressed while boiling hot. The residual cake was extracted overnight with 8 liters of ether. Upon standing the hot alcoholic filtrate deposited large quantities of light amber lipids, including crystals of cholesterol. These solids were temporarily removed, while the alcohol and ether extracts were concentrated separately. All three were eventually combined and concentrated to a volume of about 2 liters. On cooling they separated into two layers. Instead of trying to remove the last quantities of solvent which was almost impossible on account of foaming, the lipids were immediately saponified, as described later.

The extraction as carried out was not complete. To extract all the lipids several alcohol and ether extractions would have to be made. However, the product represents fairly satisfactorily the composite lipids of the brain.

*Preparation of Methyl Esters.*—The difficulties previously met in preparing the methyl esters of spleen and suprarenal fatty acids appeared to an exaggerated degree in working with brain lipids. For example in preparing the esters from the solid lipids (from benzene extraction) the following procedure was followed. 1000 gm. of lipids were placed in a 5 liter flask and dissolved by heating in 2.5 liters of 95 per cent alcohol. To this was added a solution of 500 gm. of KOH dissolved in 400 cc. of water. The mixture was refluxed for 4 hours. About 1.5 liters of the alcohol were removed by distillation until excessive foaming occurred. The residue was diluted with 2 liters of water and acidified by addition of concentrated HCl. After boiling a few minutes the

water layer was allowed to separate and was removed by siphon. More water and HCl were added to complete decomposition of the soap. Separation of the fatty acid layer was difficult. 1·liter of toluene and 50 cc. of n-butyl alcohol were added. After standing overnight a fairly good separation was effected, and the water dissolved in the fatty acids was removed by distilling off the toluene at ordinary pressure. The resultant dry acids were mixed with 2 liters of methyl alcohol containing dry HCl and refluxed for 18 hours. Upon standing overnight at ordinary temperature 100 gm. of cholesterol crystals formed and were removed by suction filtering. In a number of trials from 90 to 110 gm. of cholesterol were obtained from 1 kilo of lipids. The cholesterol thus obtained could be very easily purified by two crystallizations from ethyl alcohol, including one treatment with norit decolorizing carbon.

The alcohol-ester mixture was poured into several volumes of cold water, toluene was added, and the layers separated. The addition of n-butyl alcohol again facilitated separation. After drying the esters by toluene-distillation under reduced pressure, further distillation was impossible on account of excessive foaming. Some polymerization may have taken place at this point, because when the product was poured into methyl alcohol part of it dissolved, and the remainder settled to the bottom and was discarded. The alcohol-soluble portion was distilled successfully. The maximum yield of esters was 24 per cent of the weight of lipids used.

The lipids prepared fresh in this laboratory gave ester preparations in which it was unnecessary to make this final methyl alcohol separation. Nevertheless a considerable quantity of high boiling product remained in the distilling flask. In each preparation, therefore, a large amount of material is not included in the final product and in interpreting the results this must be remembered. The phenomenon may indicate the presence of considerable quantities of acids in the brain which easily polymerize into products that will not distil.

The results in Table I show the analysis of three preparations of methyl esters.

*Distillation of Methyl Esters.*—Esters from Lot A (to which was added a second preparation from the same lipids) and from Lot

TABLE I.

*Analysis of the Methyl Esters of the Fatty Acids of Brain.*

| | Benzene extract from desiccated brain. | Fresh brain. | | Suprarenal lipids.* |
|---|---|---|---|---|
| | Lot A. | Lot B. | Lot C. | |
| Boiling point, °C . . . . . . . . . . . | 172–243 | 183–270 | 170–245 | 170–230† |
| Pressure, *mm* . . . . . . . . . . . . . . . . | 6 | 4 | 5 | 7 |
| Molecular weight of acids . . . . . | 293.2 | 330.8 | 304.5 | 272.6 |
| Iodine No . . . . . . . . . . . . . . . | 79.22 | 96.1 | 92.6 | 60.27 |
| Polybromide No . . . . . . . . . . . . . | 10.4 | 13.2 | 16.5 | 4.3 |
| Br in bromides, *per cent* . . | 66.92 | 67.77 | 67.54 | 66.10 |
| Highly unsaturated fatty acids, *per cent* . . . . . . . | 11.5 | 14.7 | 18.4 | 5.5 |
| Melting point of bromides, °C.. | Sinter at 228 but only partly; 228–230. | | | |

\* Repeated from previous paper for comparison.

† Nearly all came over below 210°.

TABLE II.

*Results of Fractionation of Methyl Esters of Fatty Acids of Brain.*

| Fraction boiling point. | Weight. | Mean molecular weight of acids. | Iodine No. | Polybromide No. | Br |
|---|---|---|---|---|---|
| Lot A, 8 to 9 mm. pressure. | | | | | |
| °C. | *gm.* | | | | *per cent* |
| 185–194 | 52 | 268.6 | 44.90 | 2.40 | |
| 194–197 | 73 | 275.6 | 54.72 | 2.82 | 66.53 |
| 197–205 | 64 | 284.8 | 72.12 | 6.63 | 66.50 |
| 205–220 | 40 | 300.7 | 106.0 | 16.10 | 67.35 |
| 220–255 | 23 | 341.2 | 150.0 | 30.0 | 68.15 |
| Lot B, 10 mm. pressure. | | | | | |
| 190–200 | 42 | 287.4 | 54.31 | 2.6 | 67.61 |
| 200–210 | 79 | 290.7 | 74.65 | 10.3 | 67.83 |
| 210–220 | 18 | 314.2 | 109.1 | 18.82 | 67.27 |
| 220–245 | 35 | 363.3 | 147.3 | 34.21 | 68.18 |
| 245–280 | 33 | (587.7)* | 118.2 | 14.87 | 67.05 |

\* This result is no doubt in error.

B of Table I were fractionated, the results in Table II giving the analytical data on the fractions.

Two points of a descriptive nature are worthy of mention with regard to these methyl esters. When freshly prepared they were almost colorless, but even under $CO_2$ gas they rapidly darkened and finally assumed a deep reddish tinge. Also in preparing the esters in the early stages of distillation a white, ether-insoluble sublimate came over ahead of the esters. This occurred in every preparation. It settled out in the distillate and could easily be removed. Its nature was not determined.

*Preparation of Esters of Pure Highly Unsaturated Acids of Brain.*—35 gm. of ether-insoluble bromides of brain fatty acids were prepared by saponification of benzene-extracted brain lipids, liberation of the fatty acids, and direct bromination of the

TABLE III.

*Comparison of Analytical Data on Mixed Highly Unsaturated Fatty Acids of Brain and Methyl Arachidonate from Liver*

|  | Methyl arachidonate | | Esters of highly unsaturated fatty acids of brain. |
|---|---|---|---|
|  | Theory. | From liver |  |
| Boiling point, °C . | 230 (15 mm ) | 200 210 (7 mm ) | 200–258 (7 mm ) |
| Molecular weight | 318 | 312 | 339 |
| Iodine No | 319.2 | 314 1 | 327.3 |
| Refractive index |  | 1 4818 (23°) | 1.4897 (20°) |
| Polybromide No . | 301 | 77 6 | 90.0 |
| Bromine, *per cent* | 66 78 | 66.50 | 68.17 |
| Melting point, °C. |  | 228–231° | Does not melt below 250°. |

acids in cold ether. They were washed free from ether-soluble impurities by repeated treatment with cold ether with stirring each time and centrifugation. At least six very efficient washings were made in this manner. The bromides were finely ground and refluxed with 50 gm. of Zn dust for 18 hours in 150 cc. of methyl alcohol. The alcohol solution was removed, and the residue was extracted with hot alcohol. The combined alcoholic solutions were centrifugated to remove suspended material, dry HCl gas was passed into the solution, and refluxing was continued for 7 hours to complete esterification. The alcohol was mostly removed by distillation, the esters were cooled and poured into several volumes of water, and extracted with ether. The ether

was removed and the esters distilled. The yield was 5.5 gm. of nearly water-white ester which was very mobile at 0°, boiling at 200–258° at 7 mm. In order to compare this product with methyl arachidonate, the data for both are given in Table III.

## DISCUSSION OF RESULTS AND CONCLUSIONS.

Examination of the results given in Table I immediately discloses a number of important differences between the methyl esters obtained from the brain and those from the suprarenal gland. The brain esters boil over a range of 70–90°, while those from the suprarenal boil from 170–230°, only a very little coming over above 210°, a range of about 40°. The molecular weight of the acids from three brain preparations, calculated from the saponification number of esters, varied from 21 to 38 points higher than those of the suprarenal. The brain esters were also more unsaturated since the iodine numbers were 19 to 36 points higher. The iodine numbers of two of the brain preparations (from lipids prepared fresh) were higher than the theory for oleic acid (90). The most important points to be noted in these data, however, are the higher polybromide numbers of the brain esters, a higher content of bromine, 0.18 to 1.0 per cent more than the theory for methyl octobromoarachidate, and the fact that the bromides from the brain only partly melted at 228–230°. Under similar conditions bromides obtained from the esters of other glandular tissues always melted at this point.

The three preparations of esters gave 11.5 to 18.4 per cent of highly unsaturated fatty acid calculated as follows (10):

$$\text{Per cent highly unsaturated fatty acids} = \frac{\text{polybromide No.} \times 100}{\text{polybromide No. of pure esters}}$$

The polybromide number of the pure esters, as mentioned later, was found to be 90. These results are much higher than for any of the other tissues studied by this method, showing that the brain fatty acids have by far the largest per cent of highly unsaturated fatty acids of any tissues previously examined.

The results of fractionation of these esters are equally indicative of the presence of other highly unsaturated acids than arachidonic. The bromine addition products of the fractions contain from 66.50 to 68.18 per cent bromine. Those from the lower fractions sintered

and partly melted at 228–230°.   Those from the higher fractions did not melt at all but only shrank to a black mass.   It was believed at first that this behavior might be due to the presence of a mixture of brominated ester and acid.   Octobromoarachidic acid does not melt, but only blackens and chars up to 250°.   Titration of samples of these fractions with alkali, however, showed that there was never more than 1 or 2 per cent of free acid in the ester preparations, and usually less than 1 per cent.   The behavior of the bromides with heat, therefore, is not due to the presence of brominated acids.

The boiling point ranges of these two preparations of esters were approximately wide enough to cover series from $C_{16}$ to $C_{24}$, if a difference in boiling point of 15° is assumed for each difference of $C_2$ at the pressure used.   At 8 mm. pressure methyl palmitate should boil at about 180°, and methyl lignocerate at about 260°, these figures being approximations, since to the writer's knowledge they are not given in the literature.   Lignoceric acid, $C_{24}H_{48}O_2$, has been isolated from the brain by Levene (11).

The molecular weights of the acids from these fractions, calculated from the saponification number, confirm the results just mentioned.

Final proof of the presence of more than one highly unsaturated fatty acid in the brain is given in Table III.   The pure highly unsaturated esters boiled over a 58° range, gave a mean molecular weight twenty-one points too high for methyl arachidonate, an iodine number eight points too high (Wijs, 1 hour reaction) for the theory and thirteen points higher than a preparation of methyl arachidonate from the liver prepared under similar conditions, and an appreciably different refractive index.   Finally, the poly-bromide number of 90 was greater than that for methyl arachidonate and the bromine content of the bromides 1.4 per cent too high.   The bromides in this instance did not melt but only charred at 250°.

These data confirm without doubt the presence of arachidonic acid in the lipids of brain and are equally conclusive in proving the presence of another highly unsaturated acid of higher molecular weight and more unsaturated than arachidonic.   A boiling point over 250° indicates an acid of the $C_{24}$ series, and it is suggested that the acid in question may be a penta-unsaturated lignoceric

acid, the name for which would be tetracosapentenoic acid (verified by Dr. A. M. Patterson). Methyl decabromotetracosanoate should contain 68.23 per cent bromine. Found for mixed bromides, 68.17 per cent.

While the data indicate strongly the presence of these two acids, the possibility of other unsaturated acids from the $C_{20}$ to the $C_{24}$ series is not excluded. It is to be hoped that future work will disclose a method of separating and identifying more exactly the acids present.

## SUMMARY.

1. The methyl esters of the fatty acids of the lipids of beef brain have been prepared. Only 24 per cent of the total weight of lipids used can be recovered as methyl esters. A considerable amount of high boiling material cannot be distilled even under high vacuum. About 10 per cent of the weight of the lipids is recovered as cholesterol.

2. The methyl esters of the brain contain acids from the $C_{16}$ to the $C_{24}$ series. Different preparations from the brain give a content of 11.5 to 18.4 per cent of highly unsaturated acids, which is much higher than for any of the other tissues studied.

3. Evidence is presented to show that the highly unsaturated fatty acids of the brain consist of a mixture of at least two acids, arachidonic and another acid, possibly tetracosapentenoic, $C_{24}H_{38}O_2$. Other acids may be present.

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# THE STRUCTURE OF THYMONUCLEIC ACID.

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The earlier work on thymonucleic acid by Levene and Mandel[1] and by Levene and Jacobs[2] led to the isolation of monophosphoric esters of pyrimidine nucleosides and diphosphoric esters of pyrimidine nucleosides. These observations were corroborated by Thannhauser and coworkers.[3]

In a recent publication Levene and London[4] reported the isolation of a guanine nucleoside and the present paper contains a report on the isolation of three additional nucleosides, namely hypoxanthine, thymine, and cytosine nucleosides. The hypoxanthine nucleoside is undoubtedly derived from the adenine nucleoside inasmuch as the yeast nucleic acid digested in the same manner as the thymonucleic acid gave inosine in place of adenosine. The newer findings definitely confirm the formulation for the thymonucleic acid given by Levene, in 1921, which is but a modification of the one given by Levene and Jacobs, in 1912.

In one essential detail the formulation of Levene and Jacobs and of Levene needs revision; namely, the sugar entering in the structure of thymonucleic acid is now found to be not a hexose but a desoxypentose. With this correction, the formulation of the structure of thymonucleic acid becomes as follows:

[1] Levene, P. A., and Mandel, J. A., *Ber. chem. Ges.*, **41**, 1905 (1908).

[2] Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, **12**, 411 (1912).

[3] Thannhauser, S. J., and Ottenstein, B., *Z. physiol. Chem.*, **114**, 39 (1921). Thannhauser, S. J., and Blanco, G., *Z. physiol. Chem.*, **161**, 116 (1926).

[4] Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81**, 711 (1929).

$$\begin{array}{l}
\text{HO} \\
\quad\searrow \\
\text{O}\!=\!\text{P}\!-\!\text{O}\!-\!\text{C}_5\text{H}_7\text{O}\cdot\text{C}_5\text{H}_4\text{N}_5 \\
\quad\nearrow \qquad\quad | \\
\text{HO} \qquad\qquad \text{O} \\
\qquad\qquad\quad | \\
\qquad \text{O}\!=\!\text{P}\!-\!\text{O}\!-\!\text{C}_5\text{H}_7\text{O}\cdot\text{C}_5\text{H}_5\text{N}_2\text{O}_2 \\
\qquad\quad\nearrow \qquad\qquad | \\
\qquad \text{HO} \qquad\qquad \text{O} \\
\qquad\qquad\qquad\quad | \\
\qquad\qquad \text{O}\!=\!\text{P}\!-\!\text{O}\!-\!\text{C}_5\text{H}_7\text{O}\cdot\text{C}_4\text{H}_4\text{N}_3\text{O} \\
\qquad\qquad\quad\nearrow \qquad\qquad | \\
\qquad\qquad \text{HO} \qquad\qquad \text{O} \\
\qquad\qquad\qquad\qquad\quad | \\
\qquad\qquad\qquad \text{O}\!=\!\text{P}\!-\!\text{O}\!-\!\text{C}_5\text{H}_6\text{O}_2\cdot\text{C}_5\text{H}_4\text{N}_5\text{O} \\
\qquad\qquad\qquad\quad\nearrow \\
\qquad\qquad\qquad \text{HO}
\end{array}$$

$$\text{C}_{39}\text{H}_{51}\text{N}_{15}\text{P}_4\text{O}_{25}$$
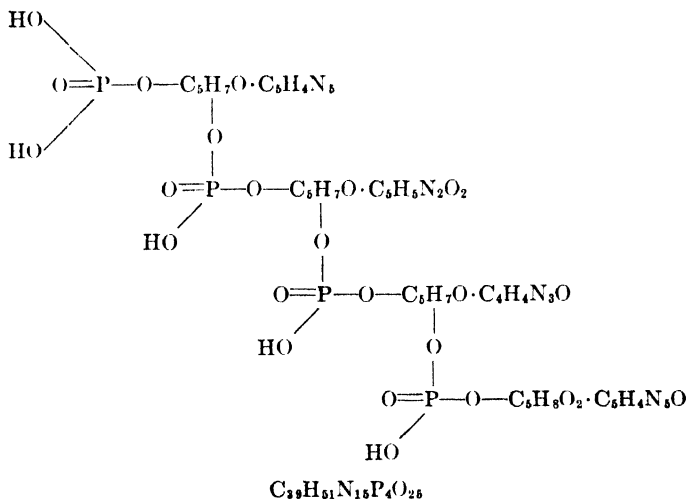
The reasons for accepting the mode of linking between individual nucleotides as given in this figure have been presented earlier. The nature of the sugar, however, needs to be discussed in some detail.

### Carbohydrate of Thymonucleic Acid.

In the communication on the guanine nucleoside it was stated that the sugar was obtained in crystalline form and that it analyzed for a desoxypentose. It gave a color test with Kiliani's reagent for 2-desoxy sugars. It did not form an osazone, but readily formed a benzylphenylhydrazone which analyzed for the hydrazone of a desoxypentose. The three new nucleosides all have the composition of derivatives of a desoxypentose. Thus there can be no doubt as to the composition of the sugar. It remains to reconcile the new findings with the old. From the work of Kossel and his school and from that of Levene,[5] it is known that the sugar of thymonucleic acid on heating with 5 to 10 per cent sulfuric acid is transformed into levulinic acid. This reaction was considered characteristic for hexoses and on this basis it had been assumed

[5] For a discussion of earlier work on the carbohydrate of thymonucleic acid see Jones, W., Nucleic acids, New York and London, 2nd edition (1920), also, Feulgen, R., Chemie und Physiologie der Nucleinstoffe, Berlin (1923).

that the sugar of thymonucleic acid was a hexose.   Levene and Mori[6] found that the reaction is equally characteristic for the synthetic 2-desoxypentoses, as well as for the sugar isolated from the thymonucleic acid.   Feulgen[7] made the important discovery that thymonucleic acid on short hydrolysis with dilute acids gave, a positive test with Schiff's reagent and a positive pine stick test. Levene and Mori found that the synthetic desoxypentoses as well as the desoxypentose of nucleic acid reacted in a similar way.

There still remain to be reconciled the analytical data for the crystalline barium salt of the diphosphoric ester of the thymine nucleoside previously described, with the new conception of its structure.   In fact, the analytical data of this substance agree better with those required by the newer theory.

$C_{11}H_{14}N_2P_2O_{13}$.   Calculated.   C 18.37, H 1.97, N 3.89, P 8.62.
$C_{10}H_{12}N_2P_2O_{11}$.        "        " 17.84, " 1.78, " 4.15, " 9.21.
                  Found.        " 18.11, " 1.93, " 4.04, " 8.97.

Thus the substance to which previously the structure of $d$-diphosphoric ester of a thymine hexoside had been assigned is in reality the diester of a thymine desoxypentoside.

There is still another point to be mentioned, namely the elementary composition of the thymonucleic acid.   The new formulation of $C_{39}H_{51}N_{15}P_4O_{25}$ requires the following elementary composition: N 16.77 and P 9.89.   It is noteworthy that the purest sample of the acid prepared by Levene and Mandel in 1908[8] contained N 17.70 and P 10.00.   These high values of the nitrogen and phosphorus content seemed at that time puzzling.

Thus the discovery of the nature of the sugar of the thymonucleic acid gives a ready explanation of those peculiarities of the acid which up to date seemed rather puzzling.

## EXPERIMENTAL.

### Introductory.

The unsuccessful attempts to find the proper conditions for partial hydrolysis of thymonucleic acid to nucleosides have led one of us (Levene) to search for conditions of enzymatic hydrolysis leading

[6] Levene, P. A., and Mori, T., J. Biol. Chem., **83**, 803 (1929).

[7] Feulgen, R., Z. physiol. Chem., **123**, 197 (1922).

[8] Levene, P. A., and Mandel, J. A., Biochem. Z., **10**, 215 (1908).

to the desired end. In cooperation with Medigreceanu,[9] he found that there occur in the organs enzymes capable of cleaving nucleic acids to nucleotides, others capable of dephosphorylating nucleotides (nucleotidases), and some capable of hydrolyzing nucleoses '(nucleosidases). The nucleotidases were found in several organs and also in the intestinal juice. The latter finding suggested the possibility of accomplishing the desired hydrolysis of nucleic acid by means of intestinal juice. In 1912, through the courtesy of Dr. Carrel,[10] Levene and Jacobs were in possession of a dog with an intestinal fistula which permitted feeding the dog on nucleic acid and collecting the nucleic acid impregnated with enzyme through the fistula. From material obtained in this manner it was then possible to isolate a small quantity of gelatinous material resembling crude guanosine which gave a negative orcinol test and which analyzed as a guanosine hexoside. The material was undoubtedly very impure and apparently contaminated with a carbohydrate which did not belong to the nucleoside inasmuch as on hydrolysis it yielded a hexosazone. In the summer of 1924 Levene performed a number of experiments in Pavlov's laboratory in Leningrad (Petrograd) Russia, attempting to bring about a satisfactory hydrolysis of thymus nucleic acid by means of intestinal juice obtained from dogs with a Thiry-Vella fistula.[11] The experiments were not successful, undoubtedly for the reason that the juice was very poor in enzymes.

The experience of 1912 seemed to suggest the advantage of passing a solution of nucleic acid through a segment of the gastrointestinal tract and collecting it from an intestinal fistula. In order to avoid contamination with remnants of food, it seemed desirable to create a gastric fistula which would permit the establishment of a clean and empty gastrointestinal segment. With this aim in view Professor E. S. London of Leningrad prepared, at the request of Levene, several dogs each with one gastric and one intestinal fistula.

[9] Levene, P. A , and Medigreceanu, F , *J. Biol. Chem ,* **9,** 65, 375, and 389 (1911).

[10] I wish to express at this late date my appreciation to Dr. Carrel for his courtesy —P. A. L.

[11] For this courtesy, I wish to express my great appreciation to Professor I. P. Pavlov. —P. A. L.

*Mode of Digestion.*

The procedure was repeatedly varied in the course of the work, and we are inclined to believe that the optimal conditions for the digestion of the nucleic acid still remain to be established. In the earlier experiments a solution of 50.0 gm. of nucleic acid was allowed to flow through the gastric fistula and the solution collected from the intestinal fistula, the entire procedure lasting from 1 to 2 hours. The volume of the collected fluid varied from 700 cc. to 350 cc. Toluene was added to the solution which was then placed in a thermostat for different intervals, the shortest being 4 days and the longest 7. The degree of digestion varied from experiment to experiment. In the later experiments small portions of gastrointestinal secretions were added daily. Towards the end of the work it was found that hydrolysis of the nucleic acid could be accomplished by adding portions of the secretions daily to the solution of nucleic acid without passing the latter through the segment of the gastrointestinal tract. This fact will permit the working out of more definite optimal conditions. Work in this direction is at present in progress in this laboratory.

The yield of nucleosides differed from experiment to experiment, the maximal being 1.5 gm. of guanine nucleoside from 200.0 gm. of nucleic acid. The other nucleosides were obtained in minimal quantities and many experiments yielded only the guanine nucleoside.

The great resistance of the thymonucleic acid as compared with yeast nucleic acid was very striking. The latter yielded about 5.0 gm. of guanosine from 200.0 gm. of nucleic acid. This fact is in harmony with observations made in 1911 by Levene and Medigreceanu.[9]

*Chemical Procedure for Isolation of Nucleosides.*

The first step in the fractionation of the digest consisted in its separation into two fractions, one yielding principally the guanine and hypoxanthine nucleosides, and the other the pyrimidine nucleosides.

The digest of 200.0 gm. of nucleic acid was poured into twice its volume of 95 per cent alcohol and the filtrate was concentrated to about 400 cc. On cooling, this solution generally gelatinized and it could be separated by filtration into the two fractions; the

gelatinous fraction serving for the isolation of the purine nucleosides, and the filtrate for the pyrimidine nucleosides. It was found expedient in order to facilitate the filtration to warm the solution of the concentrate on the boiling water bath and to add an equal volume of boiling methyl alcohol. The solution remains liquid while hot, but on cooling a voluminous precipitate is formed which is readily filtered.

*Purine Nucleoside Fraction.*

The above precipitate which contained, in addition to the nucleosides, mineral phosphates and nucleotides was dissolved in hot water and freed from phosphoric acid by means of barium hydroxide. The filtrate from the phosphates was freed from the barium ions and then concentrated to a small volume. On cooling, a semi-gelatinous precipitate formed which served for the isolation of the guanine nucleoside and a filtrate which served for the isolation of the hypoxanthine nucleoside.

*Guanine Nucleoside.*—The precipitate containing the nucleoside is dissolved in water and an excess of 25 per cent solution of basic lead acetate is added. A precipitate is formed which consists principally of nucleotides. To the filtrate an excess of ammonium hydroxide is added which causes the formation of a voluminous precipitate. The mixture is brought to a boil which causes a great part of the precipitate to dissolve. On cooling the filtrate a flocculent precipitate is formed. The mixture is allowed to stand 4 or 5 hours in the cold. The precipitate is then filtered and dissolved in water containing a little acetic acid. Hydrogen sulfide gas is passed through the solution and the filtrate from lead sulfide is concentrated under reduced pressure at a temperature of the water bath not exceeding 30°. The nucleoside then crystallizes in long needles. The substance is practically pure, but for the preparation of the desoxypentose it should be recrystallized twice out of water. The general properties of the substance have already been described, as well as its optical rotation in water. In the present paper the rotation of the other nucleosides will be given in 1.0 N sodium hydroxide and therefore the rotation of this nucleoside also has been measured in this solvent. The result was as follows:

$$[\alpha]_D^{\ast} = \frac{-0.72° \times 100}{1 \times 2} = -36.0°.$$

As was stated in the earlier publication, the nucleoside is very unstable and in order to determine the optimal conditions for its hydrolysis three experiments were performed. In each case 0.250 gm. of the substance was dissolved in 10 cc. of 0.01 N aqueous hydrogen chloride. The solutions were brought to a boil. This procedure did not consume more than 1 minute. The solution was then kept in a boiling water bath, the first 5, the second 10, and the third 15 minutes. During these intervals a white granular precipitate formed in each tube. At the end of each experiment the solutions were immersed in an ice-alcohol bath and after cooling, the filtrates were brought up to the volume of 25 cc. and the optical rotations of each were measured in a 2 dm. tube. They were as follows:

$$I \quad \alpha_D = -0.49°.$$
$$II \quad " \quad = -0.46°.$$
$$III \quad " \quad = -0.48°.$$

Inasmuch as the rotation of the desoxypentose in equilibrium is $-50°$, it follows that each solution contained 0.125 gm. of sugar. Thus it is evident that 5 minutes heating with 0.01 N hydrochloric acid suffices to bring about a complete hydrolysis of the nucleoside.

*Hypoxanthine Nucleoside.*—The solution containing this fraction was fractionated with lead in exactly the same manner as the guanine nucleoside fraction.

The hypoxanthine nucleoside crystallized in long needles and was recrystallized out of water until the substance was ash-free. The yield of this material was very small, so that all through the work only 1.0 gm. of the substance was accumulated. The air-dry substance contracted at 202° and had no melting point. It analyzed as follows:

3 860 mg. substance: 6.670 mg. $CO_2$ and 1.655 mg. $H_2O$.
3.935 " " : 0.778 cc. $N_2$ at 27° and p = 759 mm.
$C_{10}H_{12}N_4O_5$. Calculated. C 47.60, H 4.79, N 22.21.
Found. " 47.74, " 4.85, " 22.46.

The rotation of the substance in water with enough sodium hydroxide to complete solution was the following.

$$[\alpha]_D^{25} = \frac{-0.42° \times 100}{1 \times 2} = -21.0°.$$

0.500 gm. of the substance was taken up in 25 cc. of 0.01 N aqueous hydrogen chloride. The solution was brought to a boil and then allowed to remain in a boiling water bath for 10 minutes. On cooling the solution in an ice-water mixture, the base settled out. To the filtrate silver sulfate was added in excess. The silver salt of the base was removed and from the filtrate the excess of silver was removed by means of hydrogen sulfide. The solution was brought up to the volume of 25 cc. and the solution then showed in a 2 dm. tube the rotation of $-0.54°$, which corresponds to 0.250 gm. of desoxypentose, thus showing that it contained the expected amount of the sugar.

The hydrochloride of the base was redissolved in a slight excess of aqueous hydrogen chloride and the base precipitated by means of ammonia. The base was washed free from chlorides and then had the following composition.

4 475 mg substance: 1 641 cc. $N_2$ at 30° and p = 751 7 mm.
$C_5H_4N_4O$. Calculated. N 41 10. Found. N 40.82.

### *Pyrimidine Nucleoside Fraction.*

This fraction in addition to nucleosides contained phosphates, chlorides, and nucleotides. It was concentrated to a small volume and precipitated with 95 per cent alcohol as long as a precipitate formed. The filtrate was freed from phosphoric acid by means of barium hydroxide and the filtrate from the phosphates was freed from the excess of barium ions and then fractionated by means of basic lead acetate in the manner described for the guanine nucleoside.

*Thymine Nucleoside.*—The lead salts soluble on heating were used for the preparation of the nucleoside. From this fraction the lead was removed by hydrogen sulfide and the filtrate from the lead sulfide was cooled to 0° and made acid to Congo red by means of cold dilute sulfuric acid. To this solution, immersed in a cooling mixture, silver carbonate was added until all the chlorine ions were removed. The filtrate from the silver precipitate was neutralized with freshly prepared barium carbonate and through the suspension a stream of hydrogen sulfide gas was passed. The filtrate from silver sulfide and barium sulfate was freed from excess of barium ions and concentrated to a small volume and allowed to

stand in a desiccator under reduced pressure. On standing, a crystalline deposit forms consisting partly of needles and partly of platelets. This deposit consists of thymine nucleoside. For purification it is recrystallized out of water. In the course of the work we came into possession of approximately 4.0 gm. of this substance. About 2.0 gm. were used up in the attempts at hydrogenation in the same manner as the hydrogenation was carried out on uridine. The efforts thus far were not successful, but they will be continued when larger quantities of the material will be available.

The pure thymine nucleoside crystallizes in platelets. Heated in a capillary tube it melts at 185°. The composition of the substance was as follows:

5.190 mg. substance : 9.470 mg. $CO_2$ and 2.605 mg. $H_2O$.
5.100  "        "    : 0.534 cc. $N_2$ at 26° and p = 736 mm.
$C_{10}H_{14}N_2O_5$. Calculated. C 49.59, H 5.80, N 11.56.
Found.     " 49.76, " 5.64, " 11.58.

The optical rotation of the substance in 1.0 N sodium hydroxide was

$$[\alpha]_D^{25} = \frac{+0.65° \times 100}{1 \times 2} = +32.5°.$$

1.5 gm. of the substance were taken up in 1 cc. of 10 per cent sulfuric acid in a sealed tube and heated in a glycerol bath for 4 hours at 130°. On cooling, a crystalline deposit formed. It had all the properties of thymine and for analysis was recrystallized from dilute sulfuric acid. The substance was washed with water, and then with alcohol. It did not combine with the acid. The dry substance analyzed as follows:

4.330 mg. substance: 0 856 cc. $N_2$ at 30° and p = 751.7 mm.
$C_5H_6N_2O_2$. Calculated. N 22.22. Found. N 22 02.

The filtrate from thymine was extracted with ether in a continuous extractor for 60 hours. The ethereal extract contained a crystalline deposit of thymine. A similar observation had been made in the extraction of a hydrolysate of thymus nucleic acid. The crystals were removed by filtration and the residue was taken up in 5.0 cc. of water. To the solution were added 0.5 gm. of the

hydrochloride of semicarbazide and 1.0 gm. of sodium acetate dissolved in 5.0 cc. of water. On scratching with a glass rod, the semicarbazone of levulinic acid crystallized immediately. The substance was recrystallized from 98.5 per cent alcohol. The substance melted at 192° (uncorrected). The yield of recrystallized material was 0.225 gm. It had the following composition.

3.170 mg. substance: 0.692 cc. $N_2$ at 30° and p = 752 mm.
$C_6H_{11}N_3O_3$ (173). Calculated. N 24.25. Found. N 24.03.

*Cytidine Nucleoside.*—The filtrate from the thymine nucleoside was treated with an excess of a solution of alcoholic picric acid. On standing, an amorphous precipitate formed in which were imbedded microscopic balls of semicrystalline structure. The precipitate was filtered off and washed with ether. The mother liquor on concentration under diminished presure at room temperature gave another crystalline deposit. The two deposits were combined, recrystallized several times from methyl alcohol, and finally from water. The substance contracted at 190° and did not show any tendency to melt. It had the following composition.

4.885 mg. substance: 0.785 cc. $N_2$ at 25° and p = 759.
6.370 " " : 9.245 mg. $CO_2$ and 1.925 mg. $H_2O$.
$C_{15}H_{16}N_6O_{11}$. Calculated. C 39.46, H 3.53, N 18.42.
Found. " 39.57, " 3.38, " 18.38.

The optical rotation of the substance in water was as follows:

$$[\alpha]_D^{25} = \frac{+0.20° \times 100}{0.5 \times 1} = +40°.$$

Thus, in the case of the thyminose nucleosides as in that of the ribose nucleosides, the purine nucleosides rotate to the left, whereas the pyrimidine nucleosides rotate to the right.

# RIBODESOSE AND XYLODESOSE AND THEIR BEARING ON THE STRUCTURE OF THYMINOSE.

BY P. A. LEVENE AND T. MORI.*

*(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)*

This communication deals with a comparison of the properties of three desoxypentoses. One is a natural product obtained on hydrolysis of nucleosides which enter into the structure of thymonucleic acid. To this sugar provisionally the name "thyminose" will be assigned. The other two sugars are ribodesose and xylodesose. Thymonucleosides are very difficultly accessible and therefore the study of the configuration of thyminose by the analytical methods seemed of very little promise. However, the present knowledge of its properties warrants the conclusion that the sugar is a 2-desoxyaldopentose. The sugar has the elementary composition of a desoxypentose; it gives a color test with Kiliani's reagent; it gives a positive pine stick test, and on oxidation by the Willstätter and Schudel method it consumed the amount of iodine required by theory for an aldodesoxypentose; also it gives a positive test with Schiffs' reagent. There seemed to be an indication that the carbon chain of the sugar had a normal structure inasmuch as on being treated with sulfuric acid it gave rise to levulinic acid.

With this information it seemed logical to expect that a comparison of the properties of thyminose with ribodesose and xylodesose should furnish an answer to the question of the structure of the first inasmuch as only two desoxypentoses in each series are possible as seen from the accompanying figures, ribodesose had already been prepared by Meisenheimer and Jung.[1] Unfortunately these authors did not report the optical properties of the sugar, nor did they attempt to convert it to levulinic acid. Inas-

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* Fellow of the International Education Board.

[1] Meisenheimer, J., and Jung, H., *Ber. chem. Ges.*, **60**, 1462 (1927).

H   OH
 \  /
  C ———
  |       |
 OHCH  ·  |
  |       |
 HCOH     O
  |       |
 HCOH     |
  |       |
 CH₂ ———
d-Arabinose.
I.

H   OH
 \  /
  C ———
  |       |
 HCOH     |
  |       |
 HCOH     O
  |       |
 HCOH     |
  |       |
 CH₂ ———
d-Ribose.
II.

H   OH
 \  /
  C ———
  |       |
 CH₂      |
  |       |
 HCOH  ·  O
  |       |
 HCOH     |
  |       |
 CH₂ ———
d-Ribodesose
or
d-arabodesose.
III.

H   OH
 \  /
  C ———
  |       |
 HCOH     |
  |       |
 HOCH     O
  |       |
 HCOH     |
  |       |
 CH₂ ———
d-Xylose.
IV.

H   OH
 \  /
  C ———
  |       |
 HOCH     |
  |       |
 HOCH     O
  |       |
 HCOH     |
  |       |
 CH₂ ———
d-Lyxose.
V.

H   OH
 \  /
  C ———
  |       |
 CH₂      |
  |       |
 HOCH     O
  |       |
 HCOH     |
  |       |
 CH₂ ———
d-Xylodesose
or
d-lyxodesose.
VI.

804
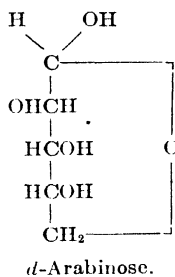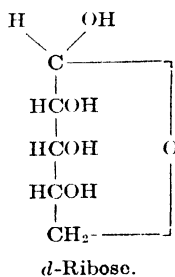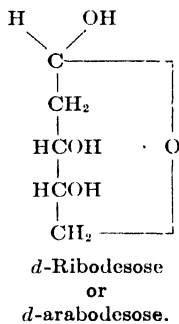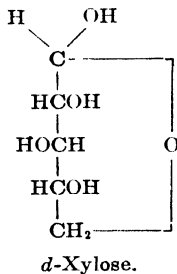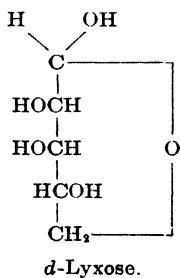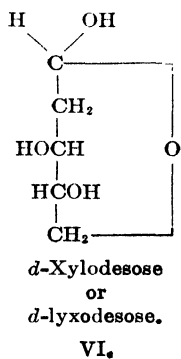
much as this knowledge was essential for us, we were obliged to synthesize this sugar.   Xylodesose has now been prepared for the first time.

To our surprise, thyminose was found to differ from both ribodesose and xylodesose.   The principal points of difference are found in the optical behavior of the three sugars as seen from the following data.

|  | d-Xylodesose. | | Thyminose. | |
|---|---|---|---|---|
|  | Water. | Pyridine. | Water. | Pyridine. |
| Initial.     ...... .. | −22 5° | −40 25° | −60 0° | −90 6° |
| Equilibrium........ | −2 0 | +50 75 | −50 0 | −40 0 |

|  | l-Ribodesose. | |
|---|---|---|
|  | Water. | Pyridine. |
| Initial. .   ...... ................. | +2.88° | +2 75° |
| Equilibrium................. ..... ........ | +2 13 | +2.13 |

In the melting point thyminose differed much from xylodesose and less from ribodesose.

| d-Xylodesose. | Mixed. | Thyminose. | Mixed. | l-Ribodesose. |
|---|---|---|---|---|
| 92° | 75° | 78° | 79° | 80.5° |

The differences were much more striking in the case of the benzylphenylhydrazones.

*Melting Points of Hydrazones.*

| d-Xylodesose. | Mixed. | Thyminose. | Mixed. | l-Ribodesose. |
|---|---|---|---|---|
| 116–118° | 104–106° | 128–130° | 115–117° | 115–117° |

*Rotations of Hydrazones in Pyridine.*

| d-Xylodesose. | Thyminose. | l-Ribodesose. |
|---|---|---|
| +13 5° | −17 5° | +7.8° |

Thus one seems to be confronted with the difficult task of the possibility of explaining that a 2-desoxyaldopentose is neither ribodesose nor xylodesose, or if thyminose is a branched chain sugar of the type of apiose, then there is the difficulty in explaining its transformation into levulinic acid.

A comparison of the optical properties of xylodesose and of thyminose excludes the possibility of the latter having the configuration of the former inasmuch as the derivative of xylose has an

initial rotation to the left and an equilibrium rotation to the right whereas thyminose rotates to the left in the freshly prepared solution as well as in the state of equilibrium.    In this respect thyminose behaves similarly to ribodesose.   The latter, however, has a very low rotation as compared with thyminose.   Is not there a possibility that arabinose in the process of transformation into the desoxy sugar is partly racemized?

The final answer to the query of the structure of thyminose will have to be postponed until such time as sufficient thyminose has been accumulated to permit its transformation into valeric acid. We request the leaving of the solution of this part of the problem to this laboratory.

A word may be said in this place on the mechanism of formation of levulinic acid from desoxyaldopentoses and from hexoses. The former transformation is very simple to conceive as a process of dehydration with a subsequent hydration.

$$
\begin{array}{cccc}
& & & \text{OH} \\
& & & \diagup \\
\text{HC}{=}\text{O} & \text{HC}{=}\text{O} & \text{H}{-}\text{C}{=}\text{O} & \text{C}{=}\text{O} \\
| & | & | & | \\
\text{CH}_2 & \text{CH}_2 & \text{CHOH} & \text{CH}_2 \\
| & | & & | \\
\text{CHOH} & \text{CHOH} & \text{CH}_2 & \text{CH}_2 \\
| & |\diagup\text{OH} & | & | \\
\text{CHOH} & \text{C} & \text{CO} & \text{CO} \\
| & |\diagdown\text{OH} & | & | \\
\text{CH}_2\text{OH} & \text{CH}_3 & \text{CH}_3 & \text{CH}_3 \\
\text{VII.} & \text{VIII.} & \text{IX.} & \text{X.}
\end{array}
$$
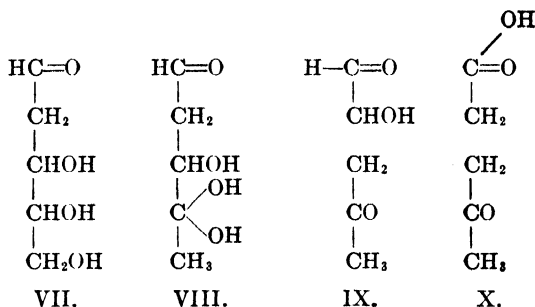
The mechanism of transformation of hexoses into levulinic acid is not fully understood although considerable attention has been given to the problem.   On the basis of the work of Kiermayer,[2] and van Ekenstein and Blanksma,[3] it is generally assumed that an early intermediate product is oxymethylfurfural.   According to Pummerer and Gump[4] this is subsequently transformed into 5-oxylevulinic aldehyde.   To this one may add that the next step

[2] Kiermayer, J., *Chem.-Ztg.*, **19**, 1004 (1895).

[3] van Ekenstein, A., and Blanksma, J. J., *Ber. chem. Ges.*, **43**, 2355 (1910).

[4] Pummerer, R., and Gump, W., *Ber. chem. Ges.*, **56**, 999 (1923).

in the chain of transformation is the conversion of the 5-hydroxy-levulinic aldehyde to 2-desoxypentose.

$$
\begin{array}{cccc}
\underset{\text{XI.}}{\begin{array}{c} \text{H} \\ \text{O=C·C} \\ \| \\ \text{HC——CH} \end{array}\overset{\displaystyle O}{\diagup}\overset{}{\diagdown}\begin{array}{c} \\ \text{C · CH}_2\text{OH} \\ \| \\ \end{array}}
&
\underset{\text{XII.}}{\begin{array}{c} \text{O  OH  OH} \\ \text{HC} \diagup \diagdown \diagup \text{C—CH}_2\text{OH} \\ | \qquad\qquad | \\ \text{H}_2\text{C————CH}_2 \end{array}}
&
\underset{\text{VII.}}{\begin{array}{c} \text{HC=O} \\ | \\ \text{CH}_2 \\ | \\ \text{CHOH} \\ | \\ \text{CHOH} \\ | \\ \text{CH}_2\text{OH} \end{array}}
&
\underset{\text{X.}}{\begin{array}{c} \overset{\text{OH}}{\diagup} \\ \text{C=O} \\ | \\ \text{CH}_2 \\ | \\ \text{CH}_2 \\ | \\ \text{CO} \\ | \\ \text{CH}_3 \end{array}}
\end{array}
$$
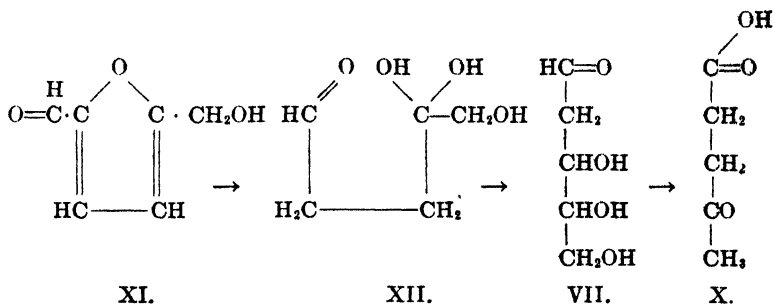
A few words may be said regarding the synthesis of desoxy-pentoses. The general procedure was the same as the one used by Bergmann and his coworkers in preparing desoxyhexoses and as used by Meisenheimer and Jung for the preparation of desoxy-arabinose. However, by carefully adhering to the details given in the experimental part, we were successful in obtaining the crystalline sugars without having to pass through the stage of their hydrazones. In this manner considerable economy is gained in time and material.

### EXPERIMENTAL.

#### *Thyminose.*

The preparation of this sugar from the guanine nucleoside has already been given by Levene and London.[5] The physical and chemical properties of the substance have not yet been described. The details of preparation as now carried out are the following. Crude substance is twice recrystallized. In this manner an ashless product is obtained. 2.0 gm. of the substance are taken up in 50 cc. of 0.01 N aqueous hydrogen chloride. The suspension is heated on a free flame until the solution is brought to a boil; generally this procedure does not last more than 1 minute. The substance dissolves into a clear solution, but soon guanine hydrochloride begins to settle out. The mixture, provided with a reflux condenser, is kept on the boiling water bath for 10 minutes and is

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[5] Levene, P. A., and London, E. S., *J. Biol Chem.*, **81**, 711 (1929).

immediately immersed in a cooling mixture. To the filtrate from the guanine hydrochloride an excess of silver sulfate is added. The excess of silver is removed by means of hydrogen sulfide and the sulfuric acid is removed quantitatively with barium hydroxide. The clear solution is concentrated under diminished pressure at room temperature. The traces of moisture are removed by repeated distillation with benzene and subsequently with alcohol. The residue is dissolved in absolute alcohol, ether is added so long as a precipitate is formed. The precipitate is generally very slight. The filtrate is then allowed to concentrate in a desiccator under diminished pressure. In the course of 24 to 48 hours the sugar crystallizes in pure form. Thus far the sugar in our possession has been so small in quantity that it has not been recrystallized. The sugar gives a positive test with Schiff's reagent. Whether this test is due to impurities or not is difficult to state. The pure and repeatedly recrystallized ribodesose gives a pronounced fuchsin coloration whereas glucodesose gives a much fainter coloration. It gives a positive test with Kiliani's reagent and a dark green coloration with a pine stick impregnated with a solution of the sugar and exposed to the vapors of aqueous hydrogen chloride. On heating in a sealed capillary tube, the substance behaves as follows: It melts into an opalescent mass at 78° and clarifies completely at about 150°.

The optical rotation of the substance was the following. In pyridine, initial $[\alpha]_D^{25} = \dfrac{-7.25° \times 100}{4 \times 2} = -90.6°$; equilibrium $= \dfrac{-3.20° \times 100}{4 \times 2} = -40.0°$. In water, initial $[\alpha]_D^{25} = \dfrac{-0.60° \times 100}{1 \times 1} = -60.0°$; equilibrium $= \dfrac{-0.50° \times 100}{1 \times 1} = -50.0°$.

During titration of the sugar by the method of Willstätter and Schudel, 0.0078 gm. of the sugar consumed 1.3 cc. of 0.1 N iodine; $C_5H_{10}O_4$ requires 1.2 cc.

*Asym.-Benzylphenylhydrazone.*

0.250 gm. of the substance was dissolved in 1 cc. of isopropyl alcohol containing 0.333 gm. of the hydrazine and the solution allowed to stand in a desiccator under reduced pressure. In 1 hour the solution turned into a solid mass. This was triturated with

isopropyl alcohol, filtered and recrystallized from isobutyl alcohol. The substance melted at 128° (uncorrected) and analyzed as follows:

4.885 mg. substance: 0.335 cc. $N_2$ at 26° and p = 762.
$C_{18}H_{22}O_3N_2$ (314.2). Calculated. N 8.91. Found. N 8.97.

The rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{-0.70° \times 100}{2 \times 2} = -17.5°.$$

*Levulinic Acid.*

4.0 gm. of guanine nucleoside were dissolved in 25.0 cc. of 10 per cent sulfuric acid and heated at 130° in a sealed tube for 4 hours. The reaction product was placed in a continuous extractor and the extraction with ether was continued for 48 hours. The ether was removed from the extract and the residue was dissolved in 5.0 cc. of water. To the solution 0.5 gm. of semicarbazide hydrochloride and a solution of 1.0 gm. of sodium acetate in 5.0 cc. of water were added. On scratching the walls of the beaker with a glass rod the semicarbazone began to crystallize immediately. After 2 hours the crystals were filtered off and recrystallized from 98.5 per cent alcohol. The substance melted at 192° (uncorrected) and had the following composition.

4.500 mg. substance: 0.953 cc. $N_2$ at 27° and p = 761 mm.
$C_6H_{11}N_3O_3$ (173). Calculated. N 24.25. Found. N 24.12.

*Xylodesose.*

*d-Acetobromoxylose.*—This was prepared by passing dry hydrogen bromide into the suspension of *d*-xylose in acetic anhydride, until saturated, according to the directions of Meisenheimer and Jung[1] for the preparation of *l*-acetobromoarabinose. The yield was 12 gm. from 10 gm. of xylose, but when lots of 100 gm. of the sugar were worked up at one time, only 90 to 100 gm. of acetobromoxylose were obtained. This method of preparation is preferable to the old one, in which glacial acetic acid saturated with hydrogen bromide was allowed to react on xylose tetraacetate. By the latter method only 70 to 80 gm. of acetobromoxylose

were obtained from 100 gm. of xylose and the preparation took more time.

*d-Diacetylxylal.*—1000 cc. of 50 per cent acetic acid in a 2 liter extraction flask were cooled with ice-salt mixture and 200 gm. of zinc dust were then added. The mixture was well agitated with a mechanical stirrer and 100 gm. of finely powdered acetobromoxylose were added in ten portions over a period of 2 hours. It was stirred for an additional hour. During all these operations, the temperature of the solution should not be higher than $-10°$. After the 3 hours, the excess of zinc was filtered off and the filtrate was diluted with twice its volume of ice water and then was extracted three times with chloroform. The combined extract, which amounted to 2000 cc., was washed first with water, then with sodium bicarbonate, and again with water. It was dried with sodium sulfate and concentrated under reduced pressure. The syrup thus obtained was fractionated at 1.5 to 1.8 mm. It boiled at 95–110°. Yield 35 gm. It was twice redistilled and the fraction distilling at 103–107° crystallized spontaneously in the form of prisms after standing for 8 days in a refrigerator at 0°. It was dissolved in very little dry ether by warming and, after cooling in an ice and water mixture, a large amount of ligroin (80–90°) was gradually added with cooling and scratching of the walls. This operation was repeated five times. The substance as thus purified had the following rotation.

$$[\alpha]_D^{25} = \frac{-9.44° \times 100}{1 \times 3.000} = -314.7° \text{ in chloroform.}$$

Further recrystallization had no effect on its optical activity.

*d*-Diacetylxylal melts at 40°[6] to an oil which solidifies again on cooling. It gives a green color when tested with HCl and a pine stick. It reduces Fehling's solution on boiling. It gives no fuchsin-$SO_2$ reaction. It is very soluble in most of the organic solvents except ligroin, in which it is sparingly soluble.

The substance analyzed as follows:

4.035 mg. substance: 7.950 mg. $CO_2$ and 2.090 mg. $H_2O$.
$C_9H_{12}O_5$.   Calculated.   C 53.96, H 6.10.
                 Found.       " 53.72, " 5.79.

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[6] All melting points in this paper are uncorrected.

0.2100 gm. of the substance in chloroform absorbed 3.3 cc. of 5 per cent bromine solution. This corresponds to 0.1650 gm. of bromine. Theory requires 0.1678 gm.

*d-Xylal.*—300 cc. of a 10 per cent barium hydroxide solution were cooled with ice-salt mixture and, when a small part of the solution was frozen, 30 gm. of diacetylxylal were added. The mixture was shaken in a machine in a cold room (8–10°) until a clear solution was obtained. Usually after 10 to 15 minutes, the temperature of the solution became 6–8° and a light yellow, clear solution was obtained. It is desirable to start from pure (but not necessarily crystalline) diacetylxylal, otherwise the solution becomes highly colored and the yield is poor. From the solution obtained as above the excess of barium was removed as barium carbonate by passing carbon dioxide into the solution and warming at 60° for about 15 minutes. The clear and practically colorless filtrate was concentrated under reduced pressure, the temperature of the bath being not higher than 30°. The syrup mixed with much barium acetate was extracted several times with absolute alcohol and the combined extract was treated with some ether and filtered clear by adding a little norit. The filtrate was concentrated under reduced pressure and the syrup obtained was evaporated several times with benzene. It was then fractionated at 1.2 mm. and boiled at 108–112°. Yield was 12 gm. It was redistilled and the middle fraction boiling at 109–112° became a crystalline mass, after being kept for about 2 weeks in the ice box. After getting one batch of crystals, we used them to seed the distillates and the crystals thus obtained were filtered from syrup in a desiccator over phosphorus pentoxide. They were then pressed between filter papers. They were dissolved in very little alcohol by warming and, after cooling, a large excess of dry ether was gradually added with scratching, whereupon xylal crystallized out in the form of glassy prisms. From the filtrate more xylal was obtained by adding petrolic ether. The substance recrystallized three times in such a way had the following rotations.

$$[\alpha]_D^{24} = \frac{-10.18° \times 100}{2 \times 2} = -254.5° \text{ in water.}$$

$$[\alpha]_D^{24} = \frac{-4.77° \times 100}{1 \times 2} = -238.5 \text{ in alcohol.}$$

*d*-Xylal melts at 49–50°. It is sweet, followed by a bitter after-taste. It is not hygroscopic after being purified and dried. It absorbs bromine. It reduces Fehling's solution but does not when diluted (1:4). It gives a fuchsin-$SO_2$ and a pine stick reaction giving a dark green coloration with the latter. It is extremely sensitive to concentrated acids, forming a dark brown precipitate, but is quite stable in alkaline solution. It is easily soluble in water, alcohol, acetone, very little in ether, and not soluble in benzene or petrolic ether.

The substance analyzed as follows:

5.085 mg. substance : 9.640 mg. $CO_2$ and 3.050 mg. $H_2O$.
$C_5H_8O_3$.    Calculated.    C. 51.71, H 6 94.
Found.          " 51 69, " 6.71.

*β-d-2-Xylodesose.*—6 gm. of crystalline xylal were dissolved in 100 cc. of 5 per cent sulfuric acid which had been previously cooled in an ice water mixture, and the solution was allowed to stand at 0°. After 5 to 10 minutes, the solution became turbid and the turbidity increased gradually, a flocculent precipitate separating after $2\frac{1}{4}$ hours. The mixture was allowed to stand for an additional $1\frac{1}{2}$ hours (altogether $3\frac{3}{4}$ hours) and was then neutralized to litmus with barium hydroxide. Most of the barium sulfate was removed by centrifugalization and the turbid, yellow filtrate was treated with a little silver sulfate solution. From the solution silver and sulfuric acid were then removed by hydrogen sulfide and barium carbonate. By this process we easily obtained a clear and less colored solution. Norit is not effective for decolorizing the solution. The clear filtrate was concentrated to a thick syrup under reduced pressure and without any heating. The syrup was evaporated several times with absolute alcohol and benzene and finally with benzene. It was taken up with 30 cc. of absolute alcohol and to this 50 cc. of dry ether were added, whereupon barium carbonate and some of the impurities were precipitated. It was filtered clear by adding a little norit and the filtrate was evaporated in a vacuum desiccator over phosphorus pentoxide and calcium chloride. The thick syrup thus obtained was kept in the ice room over phosphorus pentoxide, being scratched occasionally. After a week, well defined crystals appeared in the form of thick plates. These were mixed with cold

isopropyl alcohol and filtered, and washed several times with isopropyl alcohol until the washings were colorless. The substance thus obtained was analytically pure. The filtrate and washings were combined and treated with dry ether, again filtered with a little norit, and the filtrate was worked up just as described above. The yield of pure substance was a little more than 1 gm. From the mother liquors still more substance might have been obtained but we converted it instead into the benzyl-phenylhydrazone. The desoxy sugar can be obtained also from the xylal syrup. In this case it is best to allow it to react with sulfuric acid for $4\frac{1}{2}$ hours, though the flocculent precipitates begin to separate earlier.

The pure xylodesose shows a rapid mutarotation in dry pyridine. 20 minutes after being dissolved, it has a rotation of $[\alpha]_D^{20} = -40.25°$ and this changes to the following rotation in 30 hours, having then reached equilibrium.

$$[\alpha]_D^{22} = \frac{+ 2.03° \times 100}{2 \times 2} = + 50.75° \text{ in dry pyridine.}$$

In water it mutarotates more rapidly than in pyridine. The substance was dissolved in water cooled with ice and the rotation was observed as quickly as possible. The specific rotation was $[\alpha]_D^{22} = -22.5°$ 4 minutes after dissolving. The equilibrium was reached in 20 minutes and the rotation was then

$$[\alpha]_D^{22} = \frac{- 0.04° \times 100}{2 \times 1} = - 2.0° \text{ in water.}$$

The above change of rotation was not due only to the change in temperature, as was proved by the following experiment. The xylodesose was dissolved in water of room temperature and the rotation changed from an initial $[\alpha]_D^{22} = -6.0°$ to $-2.0°$ on standing. $\beta$-$d$-2-Desoxyxylose melts at 92–96°. It gives the fuchsin-$SO_2$ and pine stick reaction, but neither the orcinol nor aniline acetate test for ordinary pentose. It reduces Fehling's solution. It is very sensitive to concentrated acid, separating a humous substance. It has a pleasant, sweet taste, though not strong. It is soluble in water, pyridine, alcohol, slightly soluble in isopropyl

alcohol and acetone, but insoluble in ether, chloroform, benzene, and carbon tetrachloride.

0.265 gm. of pure desoxyxylose consumed 4.2 cc. of 0.1 N iodine solution by Willstätter and Schudel's method while theory requires 4.0 cc.

The substance analyzed as follows:

5.445 mg. substance: 8.975 mg. $CO_2$ and 3.050 mg. $H_2O$.
$C_5H_{10}O_4$.  Calculated.  C 44.75, H 7.51.
Found.       " 44.94, " 7.39.

*d-2-Xylodesose-A sym.-Benzylphenylhydrazone.*—0.2 gm. of pure xylodesose was dissolved in 1 cc. of isopropyl alcohol and 0.26 gm. of freshly distilled benzylphenylhydrazine was then added. After a few hours in a desiccator over calcium chloride, the mixture had changed to a crystalline mass. It was recrystallized from isopropyl alcohol. It has the following rotation.

$$[\alpha]_D^{25} = \frac{+\ 0.54° \times 100}{1 \times 4} = +\ 13.5° \text{ in dry pyridine.}$$

In starting from syrupy xylodesose contaminated with a little xylal, it was found preferable to add an equal weight of hydrazine to the syrup. After standing over calcium chloride in an ice room and scratching occasionally, the benzylphenylhydrazone crystallized in a few days. It was recrystallized first from isopropyl alcohol and then from 40 per cent alcohol, altogether six or seven times. The hydrazone thus obtained had the same rotation as above, $[\alpha]_D^{25} = +13.5°$ in dry pyridine (concentration was 2 per cent).

*d*-2-Xylodesose benzylphenylhydrazone melted at 116–118° and solidified again on cooling. It crystallizes in the form of white prisms from isopropyl alcohol and in plates from dilute alcohol. It is easily soluble in pyridine, alcohol, acetone and slightly soluble in cold isopropyl alcohol or ether, but is insoluble in water

The substance analyzed as follows:

4.465 mg. substance: 11.295 mg. $CO_2$ and 2.745 mg. $H_2O$.
5.995  "       "      :  0.470 cc. N (752 mm., 20°).
$C_{18}H_{22}N_2O_3$.  Calculated.  C 68.97, H 7.06, N 8.94.
Found.       " 68.98 " 6.88. " 9.02.

*β-l-2-Ribodesose.*—This has been already prepared by Meisenheimer and Jung[1] but they do not record the optical rotation. For this reason we repeated their experiment.

*l*-Acetobromoarabinose was reduced in the same way as done by Gehrke and Aichner.[7] This was deacetylated and by careful working we easily obtained crystalline arabinal without distillation. After being crystallized from boiling benzene, we found it had the same melting point and optical rotation as given by Meisenheimer. *l*-Arabinal was then converted to the desoxy sugar by 1.0 N sulfuric acid. After removing the sulfuric acid with barium hydroxide and concentrating the filtrate without heating, we took up the syrup with absolute alcohol and treated it with about twice its volume of dry ether. It was filtered and the filtrate was evaporated over phosphorus pentoxide and calcium chloride. In this way the *l*-ribodesose was obtained in crystalline form without difficulty. The optical rotations of the analytically pure substance were as follows: 10 minutes after dissolving in dry pyridine $[\alpha]_D^{23} = +2.75°$, and at equilibrium (after 18 hours)

$$[\alpha]_D^{23} = \frac{+0.17° \times 100}{2 \times 4} = +2.13° \text{ in dry pyridine.}$$

In water it also mutarotates. From $[\alpha]_D^{23} = +2.88°$ the rotation was changed in 18 hours to the following.

$$[\alpha]_D^{23} = \frac{+0.17° \times 100}{2 \times 4} = +2.13° \text{ in water.}$$

*β-l*-2-Ribodesose melts at $80.5 - 90°$. 0.0500 gm. of the substance consumed 7.5 cc. of 0.1 N iodine solution, while the theory requires 7.45 cc. The substance analyzed as follows:

5 890 mg. substance: 9.705 mg. $CO_2$ and 3 940 mg. $H_2O$.
$C_5H_{10}O_4$. Calculated. C 44.75, H 7.51.
                 Found.      " 44.93, " 7.48.

*l-2-Ribodesose-Asym.-Benzylphenylhydrazone.*—This was prepared from pure ribodesose and recrystallized from isopropyl alcohol. The optical rotation was

$$[\alpha]_D^{25} = \frac{+0.47° \times 100}{2 \times 3} = +7.8° \text{ in dry pyridine.}$$

[7] Gehrke, M., and Aichner, F. X., *Ber. chem. Ges.*, **60**, 918 (1927).

It melts at 115–117° in a sealed capillary to a colorless oil and does not crystallize again on cooling.

The substance analyzed as follows:

5 325 mg. substance: 13 500 mg. $CO_2$ and 3.425 mg. $H_2O$.
5.835 "        "    :   0.451 cc. N (765.8 mm., 25°).
$C_{18}H_{22}N_2O_3$. Calculated.  C 68.97, H 7 06, N 8.92.
Found.        " 69 13, " 7.19, " 8.91.

### *Levulinic Acid.*

1.2 gm. of ribodesose were dissolved in 5.0 cc. of 10 per cent sulfuric acid. To the solution 0.050 gm. of norit were added and the treatment continued exactly as in the case of the guanine nucleoside. The semicarbazone had a melting point of 192° (uncorrected) and had the following composition.

4 865 mg. substance: 1.039 cc. $N_2$ at 29° and p = 759 mm.
$C_6H_{11}N_3O_3$ (173)   Calculated.  N 24.25.  Found.  N 24.10.

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